THE UNIVERSITY OF QUEENSLAND

AUSTRALIA

Enhancing algal biomass and biofuels recovery from open culture systems

Xue Bai

A thesis submitted for the degree of Doctor of Philosophy at

The University of Queensland in 2015

School of Chemical Engineering

Abstract

Growth of algae for the purposes of generating biofuels and/or other bioproducts holds promise due to numerous advantages unique to algae. But there are serious barriers to commercial scale algae cultivation and downstream processing. Two barriers addressed in this thesis are: (i) carbon limitation in open pond algal cultivation, and (ii) difficulties for downstream processing due to the robust nature of algal cells. Hence the overall aims of this thesis were to:

- Understand and model the contribution of carbon cycling by heterotrophic bacteria to algal growth, and
- Facilitate effective downstream processing by applying a novel free nitrous acid (FNA) pre-treatment to disrupt algal cells.

To understand the contribution of heterotrophic bacteria to algal growth, carbon cycling driven by bacteria was evaluated in open algal culture systems. Since pH controls the proportion of carbon in the various inorganic pools, two different pHs were trialled. The contribution of bacteria to carbon cycling was determined by quantifying algae growth with and without supplementation of bacteria. It was found that adding heterotrophs to an open algal culture dramatically enhances algae productivity. Increases in algae productivity due to supplementation of bacteria of 4.8 and 3.4 times were observed in two batch tests operating at two different pH values over 7 days. A simplified kinetic model was proposed which described carbon limited algal growth. Simulation of the model highlighted how carbon limitation can be overcome by bacteria re-mineralising photosynthetic end products. The model was extended into a full Algae-Bacteria Model (ABM) to describe carbon, oxygen and nitrogen flows in open algal systems. The integrated model, presented in an easy to use Petersen's matrix format, provides the first description of algal cultivation processes considering the effect of heterotrophic bacteria driven carbon cycling.

The second aim of the thesis was addressed through the development and use of a novel pre-treatment technology – FNA pre-treatment – to enhance biofuels production, including (i) enhancing lipids, and specifically triacylglycerides (TAG) recovery for biodiesel production, and (ii) enhancing methane yield during anaerobic digestion of algae.

For enhancing lipids, and specifically TAG recovery, laboratory batch tests, with a range of FNA conditions and pre-treatment time, were conducted to disrupt algal cells prior to lipid extraction by

organic solvents. Total lipid (TL) quantified by the Bligh and Dyer method was found to increase with longer pre-treatment time (48 h) and higher FNA concentration (up to 2.19 mg HNO_2-N L^{-1}). Lipid extraction kinetic analysis was also conducted by Soxhlet extraction apparatus. The mass transfer coefficient (*k*) for lipid extraction from algae treated with 2.19 mg HNO_2-N L^{-1} FNA was found to increase dramatically to 0.96 h^{-1} over the untreated algae (0.39 h^{-1}). The quantity of TAG among total lipid recovered was boosted with increasing FNA pre-treatment up to an FNA concentration of 2.25 mg HNO_2-N L^{-1}. But higher FNA concentrations (13.49 and 26.98 mg HNO_2-N L^{-1}) were detrimental to polyunsaturated fatty acid (PUFA) recovery, which resulted in a decrease in TAG recovery.

For enhancing methane yield during algal digestion, the feasibility of using FNA pre-treatment was investigated through laboratory biochemical methane potential tests. It was demonstrated that methane production was dramatically enhanced by FNA pre-treatment (2.31 mg HNO_2-N L^{-1}), with a 53 % increase in the methane yield (from 161 to 250 L CH_4 per kg VS). A two substrate model was used to describe digestion to account for the apparent presence of rapid and slowly degradable material. Model-based analysis revealed that with FNA pre-treatment, the availability of both rapid and slowly biodegradable substrates were increased.

Declaration by author

This thesis is composed of my original work, and contains no material previously published or written by another person except where due reference has been made in the text. I have clearly stated the contribution by others to jointly-authored works that I have included in my thesis.

I have clearly stated the contribution of others to my thesis as a whole, including statistical assistance, survey design, data analysis, significant technical procedures, professional editorial advice, and any other original research work used or reported in my thesis. The content of my thesis is the result of work I have carried out since the commencement of my research higher degree candidature and does not include a substantial part of work that has been submitted to qualify for the award of any other degree or diploma in any university or other tertiary institution. I have clearly stated which parts of my thesis, if any, have been submitted to qualify for another award.

I acknowledge that an electronic copy of my thesis must be lodged with the University Library and, subject to the General Award Rules of The University of Queensland, immediately made available for research and study in accordance with the *Copyright Act* 1968.

I acknowledge that copyright of all material contained in my thesis resides with the copyright holder(s) of that material. Where appropriate I have obtained copyright permission from the copyright holder to reproduce material in this thesis.

Publications during candidature

Xue Bai, Paul A. Lant, Steven Pratt **(2014)**. The contribution of bacteria to algal growth by carbon cycling. *Biotechnology and Bioengineering.* doi: 10.1002/bit.25475.

Xue Bai, Forough Ghasemi Naghdi, Liu Ye, Paul A. Lant, Steven Pratt **(2014)**. Enhanced lipid extraction from algae using free nitrous acid pre-treatment. *Bioresource Technology*. 159: 36-40.

Xue Bai, Peer M. Schenk, Zhiguo Yuan, Paul A. Lant, Steven Pratt **(2015)**. Enhanced triacylglyceride extraction from microalgae using free nitrous acid pre-treatment. *Applied Energy. (accepted subject to revision).*

Xue Bai, Paul A. Lant, Paul D. Jensen, Sergi Astals, Steven Pratt **(2014)**. Enhancing methane production from algae by free nitrous acid pre-treatment *(Submitted).*

Jessica J. Buchanan, Frances R. Slater, ***Xue Bai***, Steven Pratt. (**2012**). Algal growth and community structure in a mixed-culture system using coal seam gas water as the water source. *Environmental Technology*. 34(6): 695-701.

Publications included in this thesis

Xue Bai, Paul Lant, Steven Pratt **(2014)**. The contribution of bacteria to algal growth by carbon cycling. *Biotechnology and Bioengineering.* doi: 10.1002/bit.25475.

X Bai operated all the experiments. P Lant, and S Pratt advised on the experimental design. P Lant and S Pratt critically reviewed the paper.

The research objectives, approach and outcome of this paper are included in Chapter 3-5 and the paper is wholly attached in the Appendix A.

Xue Bai, Forough Ghasemi Naghdi, Liu Ye, Paul Lant, Steven Pratt (**2014**). Enhanced lipid extraction from algae using free nitrous acid pretreatment. *Bioresource Technology*. 159: 36-40.

X Bai operated the experiments of algae cultivation and harvesting, free nitrous acid (FNA) pre-treatment and Bligh and Dyer lipid extraction. X Bai and F Ghasemi Naghdi conducted the Sohxlet lipid extraction experiments jointly. L Ye, P Lant, and S Pratt advised on the experimental design. P Lant and S Pratt critically reviewed the paper.

The research objectives, approach and outcome of this paper are included in Chapter 3-5 and the paper is wholly attached in the Appendix C.

Xue Bai, Peer M. Schenk, Zhiguo Yuan, Paul A. Lant, Steven Pratt **(2015)**. Enhanced triacylglyceride extraction from microalgae using free nitrous acid pre-treatment. *Applied Energy. (accepted subject to revision).*

X Bai operated the experiments of algae cultivation and harvesting, free nitrous acid (FNA) pre-treatment and total lipid extraction. P Schenk supported for fatty acid profile characterisation. Z Yuan, P Schenk, P Lant, and S Pratt advised on the experimental design and critically reviewed the paper.

The research objectives, approach and outcome of this paper are included in Chapter 3-5 and the accepted paper is wholly attached in the Appendix D.

Xue Bai, Paul Lant, Paul Jensen, Sergi Astals, Steven Pratt **(submitted 07/2014)**. Enhancing methane production from algae by free nitrous acid pre-treatment. *Renewable Energy.*

X Bai operated the experiments of algae cultivation and harvesting, free nitrous acid (FNA) pre-treatment and biochemical methane potential tests. X Bai and S Astals conducted the two-substrate model analysis. P Lant, P Jensen and S Pratt advised on the experimental design and critically reviewed the paper.

The research objectives, approach and outcome of this paper are included in Chapter 3-5 and the submitted paper is wholly attached in the Appendix E.

Contributions by others to the thesis

This thesis includes contributions made by others, particularly in the chemical analysis of wastewater and reactor samples. These contributions are acknowledged as follows:

Dr. Beatrice Keller-Lehmann, Jianguang Li and Nathan Clayton operated Flow Injection Analyzer (FIA) for ammonia, nitrate, nitrite, and phosphate detection and the Total Organic Carbon Analyser for total inorganic carbon (TIC) detection.

Statement of parts of the thesis submitted to qualify for the award of another degree

None.

Acknowledgements

I would like to thank my advisors Prof. Paul Lant and Dr. Steven Pratt, for their kindness, patience and most important academic excellence. I am grateful for their guidance, ever available to provide feedback and to have discussions with me, and for always having the faith in me to develop and explore my ideas. Both of their enthusiasm for research has been always an inspiration and their ability to structure and refine documents impressed me every time. Steven was always available to talk with me with my problems; and Paul helped me to step back to view the whole picture and provide me intelligent advice in many different aspects. I am sincerely grateful for the opportunity to work with them.

I would also like to thank Prof. Zhiguo Yuan and Dr. Liu Ye for their promotion of FNA techonogy for cell disruption. I am also grateful to Prof. Peer Schenk who provided valuable insights and research resources to my research. I thank the staff at the School of Chemical Engineering and AWMC for helping with all administrative and analytical tasks.

I am grateful to the students who I have worked with in my project. Philip, who is always willing to discuss with me and helped me finding my way out. Forough, who is more than a good friend to me, was always available to help me with my problems; both those relating to the PhD and personal ones. A big thank you to Monica, Liliana, Wei and the people from Prof. Schenk's lab, who are always open to discuss and talk.

It has been a pleasure for me working with the people down in Chem Eng PhD room. You have been a great group of friends who have helped me to get through the thesis by creating such an enjoyable working environment. It has been a pleasure working with you and I can only hope that my next workplace will be full of people as great as you.

I am also grateful to the financial support from China Scholarship Council and the School of Chemical Engineering for topping me up the scholarship.

Importantly, I would give a big hug to my dear parents who are in China but never stopped giving me love and support. I would also like to thank my friends who are far away and scattered around Australia and the world but have never failed to send their love and support.

Now I am finally at the end of the PhD track. Another journey just begins.

Keywords

algae, bacteria, model, free nitrous acid, lipid, triacylglyceride, anaerobic digestion, methane

Australia and New Zealand Standard Research Classifications (ANZSRC)

100302 Bioprocessing, Bioproduction and Bioproducts, 30 %

090703 Environmental Technologies, 30 %

090499 Chemical Engineering not elsewhere classified, 40 %

Fields of Research (FoR) Classification

0904 Chemical Engineering, 100 %

Table of contents

Nomenclatures

ABM	Algae-Bacteria Model
AD	Anaerobic Digestion
ASM	Activated Sludge Models
BMP	Biochemical Methane Potential
CA	Carbonic Anhydrase
CCMs	Carbon Concentrating Mechanisms
CN	Cetane Number
DAF	Dissolved Air Flotation
DIC	Dissolved Inorganic Carbon
DU	Degree of Unsaturation
DW	Dry Weight
EF	Electrolytic Flotation
EFAs	Essential Fatty Acids
FAME	Fatty Acid Methyl Ester
FIA	Flow Injection Analyzer
FNA	Free Nitrous Acid
HRAP	High Rate Algal Pond
MaB-floc	Microalgal Bacterial Floc
MIMS	Membrane Inlet Mass Spectrometer
MUFA	Mono Unsaturated Fatty Acid
OD	Optical Density
PUFA	Polyunsaturated Fatty Acid
RWQM1	River Water Quantity Model No.1
$SCCO_2$	Supercritical Carbon Dioxide Extraction
SCOD	Soluble Chemical Oxygen Demand
SFA	Saturated Fatty Acid
STKN	Soluble Total Kjeldahl Nitrogen
TAG	Triacylglyceride
TC	Total Carbon
TCOD	Total Chemical Oxygen Demand
TIC	Total Inorganic Carbon
TKP	Total Kjeldahl Phosphorous
TKN	Total Kjeldahl Nitrogen
TL	Total Lipid
TS	Total Solids
VS	Volatile Solids
WAS	Waste Activated Sludge
WWTP	Wastewater Treatment Plant

List of Figures

List of Tables

1 INTRODUCTION

1.1 Background

It has been estimated that about 90% of our current energy consumption is provided by fossil fuels, with less than 10% coming from renewable energy sources (Chen et al., 2011a; Yen et al., 2013). But the contribution of renewables, including biofuels, is growing. This thesis focuses on biofuel derived from algae. Algal-derived biofuels hold promise for the following reasons,

- Salt or brackish water and wastewater can be used to cultivate algae, which means avoiding additional demands on freshwater supplies (Chisti, 2007; Park et al., 2011).
- Algae can be cultivated using CO_2 that is released from power plants (Lardon et al., 2009; Sander & Murthy, 2010; Yang et al., 2011).
- Yields of microalgae are high compared to terrestrial plants (Chen et al., 2011a; Chhetri & Islam, 2008; Wijffels & Barbosa, 2010).
- The lipid content of many algae species is relatively high (Li et al., 2008; Rao et al., 2007; Richmond, 2004; Yeesang & Cheirsilp, 2011; Yoo et al., 2010).
- Additional to being a feedstock for biofuel production, algal biomass has value as human food supplement, animal feed, and a source of high-value bioactives (Metting, 1996; Spolaore et al., 2006). Therefore, algae offer a multiple-benefit opportunity.

Despite plenty of advantages of using algal biomass for producing bioenergy and bioproducts, several bottlenecks in the algal biorefinery pipeline restrict this technology from competing with other technologies. This thesis addresses two of these bottlenecks.

1.2 Thesis Objectives – General

This thesis is divided into two parts. These being: (i) modelling of open algal mass cultivation systems, with emphasis on the contribution of bacteria to carbon cycling, and (ii) pre-treatment of harvested biomass to enhance downstream processing.

The general aims of these two research components are:

1. To understand and model the contribution of carbon cycling by heterotrophic bacteria to algal growth, and,
2. Facilitate effective downstream processing by applying a novel free nitrous acid (FNA) pre-treatment to disrupt algal cells.

1.3 Thesis Outline

This first chapter provides a brief introduction and documents the organisation of the thesis. It outlines the two main areas investigated in this study.

Chapter 2 is a review of research on open algal culture systems. The key bottlenecks are identified in the chapter, with special attention paid to the two areas chosen for investigation, namely: (i) the contribution of carbon cycling by heterotrophic bacteria to algal growth, and (ii) pre-treatment to enhance downstream processing.

The research objectives are outlined in Chapter 3. They are:

Part I: Understanding and modelling the contribution of carbon cycling by heterotrophic bacteria to algal growth

- Research Objective 1 (RO1) – Understand and model the contribution of bacteria to algal growth by carbon cycling in lab-scale algal culture system

Part II: Pre-treatment of harvested algae to enhance downstream processing

- Research Objective 2 (RO2) – Enhance lipid, and specifically triacylglycerides (TAG), extraction from algae by using free nitrous acid pre-treatment

- Research Objective 3 (RO3) – Enhance methane production from algal digestion by free nitrous acid pre-treatment

The experimental approach is outlined in Chapter 4. And the outcomes of the research are summarised in Chapter 5. Conclusions and opportunities for future research are in Chapter 6.

2 LITERATURE REVIEW

This chapter reviews research on algal cultivation systems and identifies key bottlenecks. Section 2.1 provides an introduction to algal bioenergy production. Section 2.2 goes on to present the algal biofuel production technology train and identifies several key hurdles preventing it from becoming economically viable. Section 2.3 and 2.4 provide the detailed background leading in to the research objectives chosen for investigation in this thesis.

2.1 Introduction

The concept of using algae to produce biofuels has already been proposed for more than 50 years (Golueke & Oswald, 1959; Myers et al., 1951). Algae are small sunlight-driven factories that can convert CO_2 to biofuels such as lipids and biohydrogen; as well as a number of other valuable pharmaceutical and nutraceutical products (Metting & Pyne, 1986; Metzger & Largeau, 2005; Singh & Olsen, 2011; Singh & Gu, 2010). In addition, these photosynthetic microorganisms are useful in bioremediation applications (Muñoz & Guieysse, 2006) and some even fix nitrogen (Vaishampayan et al., 2001).

Biofuel production from algae biomass could involve one or a combination of four main pathways (Figure 2.1) (Amin, 2009; Beer et al., 2009; Brennan & Owende, 2010; Craggs et al., 2011; Schenk et al., 2008): (i) biohydrogen produced during biochemical processes of algae; (ii) fermentation of algae carbohydrates to ethanol or butanol; (iii) extraction and transesterification of algae lipid triacylglycerides to produce biodiesel; and (iv) anaerobic digestion of harvested algae biomass to produce biogas (methane).

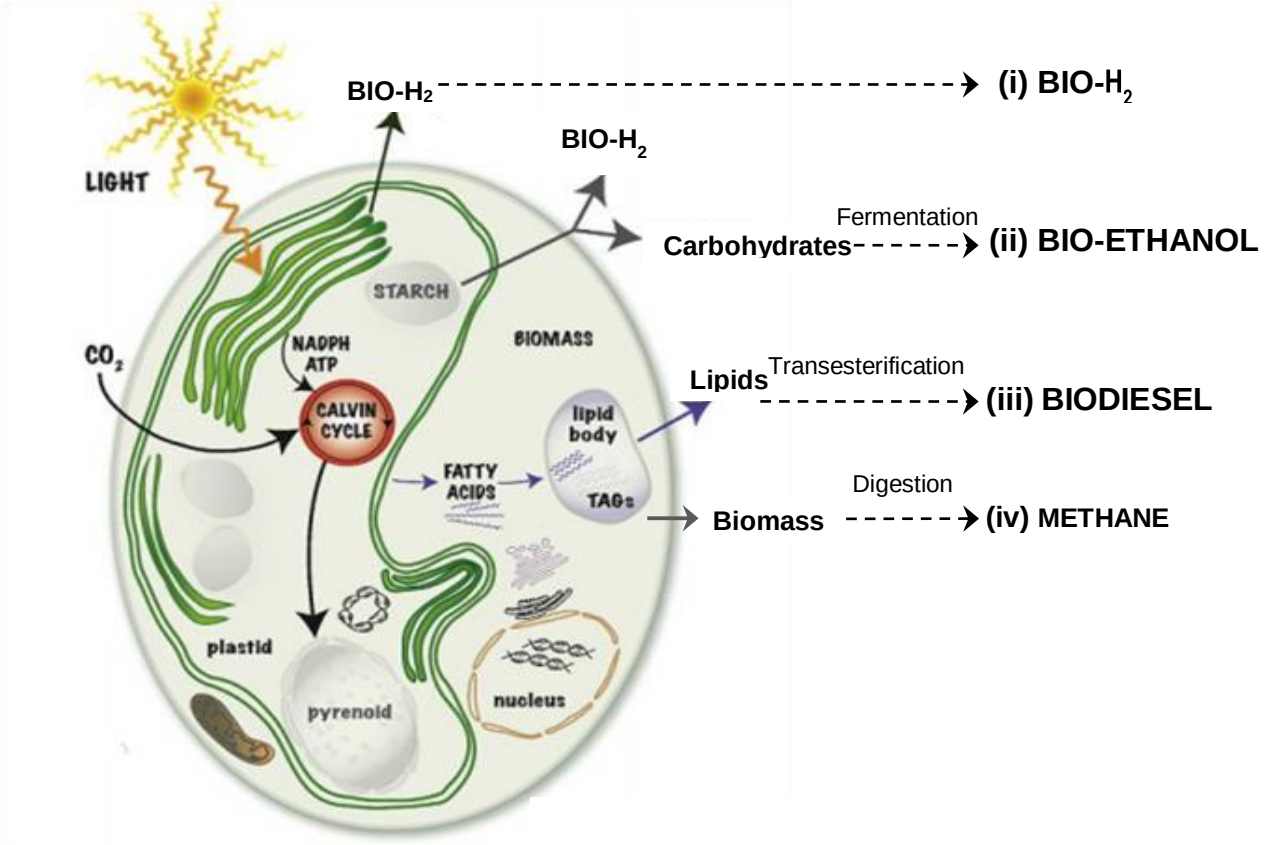


Figure 2.1 Four major types of biofuels produced from algae. Figure modified from (Beer et al., 2009).

Plant or algal oil, which contains triacylglycerides, is used to make biodiesel via the transesterification process. However, unlike the other oil crops, algae grow extremely rapidly and many are rich in oil. Algal biodiesel can be easily compared to fossil diesel because they have similar physical properties and calorific value (Xu et al., 2006). Thus algal biodiesel behaves similarly when combusted in a regular diesel engine. This merit makes algal biodiesel suitable for replacing conventional fossil diesel, instead of redesigning the whole fossil diesel engine system. This is an advantage of algal biodiesel over other renewables such as solar or biohydrogen. In addition, anaerobic digestion may be coupled with biodiesel production for utilization of post-extracted algal biomass to increase the entire energy output from algal biomass.

Biogas (methane) produced from algal biomass or residue via anaerobic digestion could potentially become a necessary step to make algal biodiesel viable (Sialve et al., 2009). Sialve et al. (2009) considered nitrogen and phosphorus remineralisation using anaerobic digestion and moreover to recover additional energy through methane. Detailed analysis was made by the authors that the conversion of algal biomass after lipid extraction into methane that can recover more energy than the

energy from the cell lipids.

The digestion process can be briefly described as a process in which organic substrates (in this case algal biomass) are broken down by microorganisms under anaerobic conditions, which leads to the production of biogas (containing mainly methane and carbon dioxide). In theory, the maximum methane yield from algae can be around 800 L kg VS^{-1}, which is calculated from the composition (given by $CO_{0.48}H_{1.83}N_{0.11}P_{0.01}$) of algal biomass (Bohutskyi & Bouwer, 2013; Sialve et al., 2009).

All the above advantages make algae a very promising candidate for biofuel production. However, like most new energy production technologies, algal biofuel production is still some way from being commercially viable or carbon neutral. This thesis aims addressing several of the challenges.

2.2 Current technologies and opportunities for process enhancement

It has been widely discussed that although there are a number of alternatives for generating energy from algae, biodiesel and methane should be the main focus (Chisti, 2008; Hu et al., 2008; Scott et al., 2010). Figure 2.2 depicts a conceptual diagram of the algal biodiesel and methane production. The figure also defines the research objectives which are investigated in this thesis. At each stage, there are many factors to be considered and optimized, including material and energy inputs (*eg.* CO_2 supplementation, energy consumption for lipid extraction) and appropriate treatment of waste products (*eg.* spent media and residual biomass). These bottlenecks in the algal biofuel production pipeline (outlined below) need to be addressed to make such technology feasible and widely adoptable. In the following sections (Section 2.3 and 2.4) a more detailed review of two key areas addressed in thesis (highlighted in Figure 2.2) is provided.

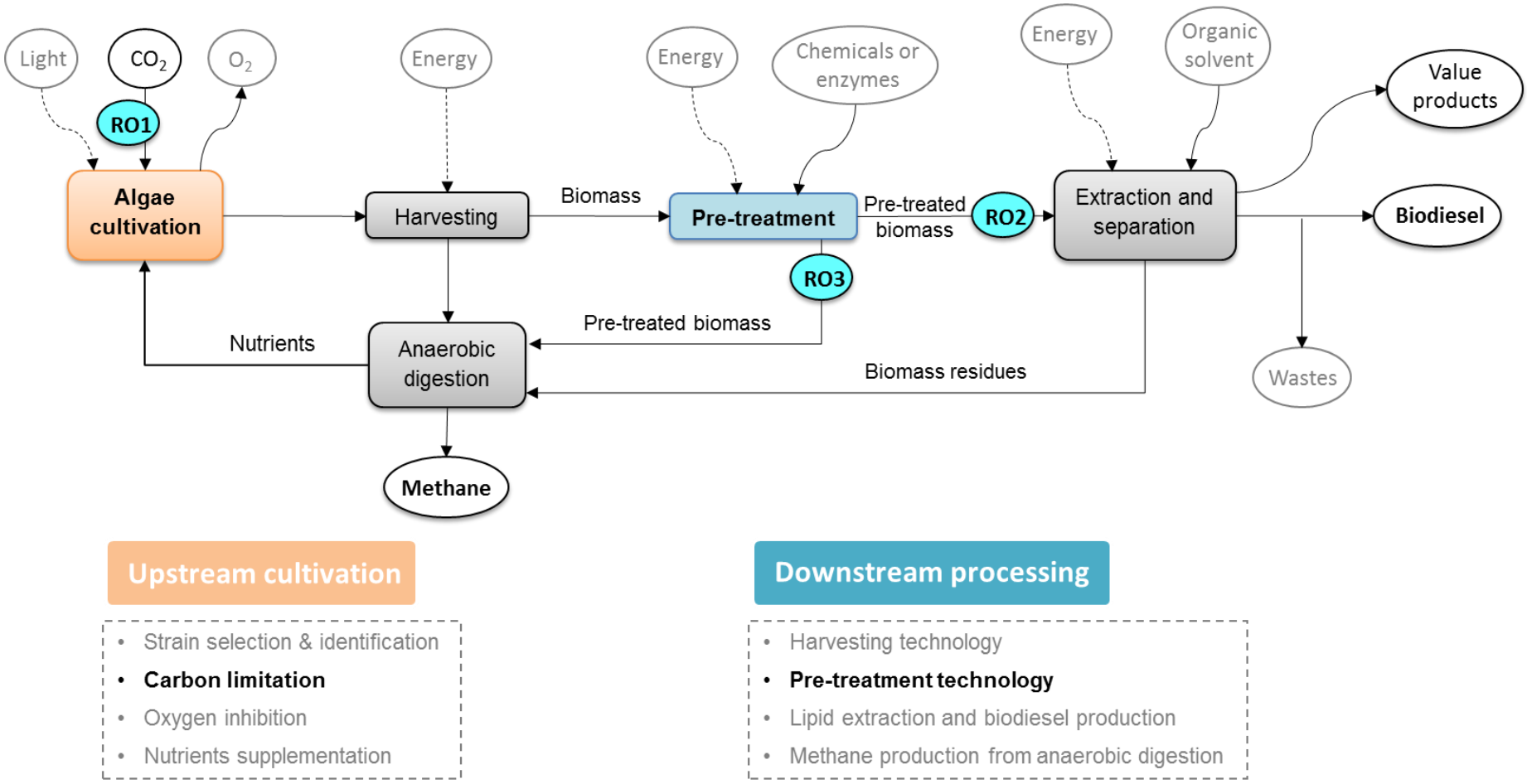


Figure 2.2 Conceptual flow diagram of current algal biodiesel and methane production pipeline, showing the major stages in the process, together with the inputs and outputs that must be taken into consideration. Opportunities for enhancement in upstream and downstream processes are also shown. Cyan highlighted processes show the research objectives to be addressed in this thesis.

2.2.1 Algal cultivation

The growth of algal biomass is an essential step in the biofuel production pipeline. Extensive work has been performed in this area focusing on design of growth system (Chen et al., 2011a; Cohen et al., 1988; Rodolfi et al., 2009). Up to now, there are three types of algal growth systems, defined by whether or not the algal culture is directly exposed to the atmosphere: open system, closed system, and hybrid system (a combination of both closed and open system) (Brennan & Owende, 2010; Chisti, 2007; Gallagher, 2011; Ho et al., 2010; Schenk et al., 2008). Considering the feasibility for process scale-up and operation costs, open systems beat closed systems (Das et al., 2011; Weissman et al., 1988). This is reflected in the number of commercial open and closed cultivation systems. Well over 90% of the world's commercial algae production uses open systems. Closed PBRs represent only about 10% of total commercial algae production (Chisti, 2007).

There are an estimated 300 000 algae species, whose diversity is much greater than that of terrestrial plants (Richmond, 2004). Currently, research work of strain selection mainly focuses on algae species which have been found to be able to (i) grow fast, (ii) accumulate substantial quantities of lipid or high value products (*e.g.* astaxanthin from *Haematococcus*), and (iii) effectively remove nutrients in wastewater. For open systems maintaining any particular algal strain is difficult unless extremophiles (*e.g.* salt tolerant algae) are used (Buchanan et al., 2012).

Table 2.1 summarizes the typical studied algal species which has relatively high biomass yield, lipid content or methane potential via anaerobic digestion. The sampling and selection process is well established, although it requires specialized equipment and may be time consuming (Richmond, 2004).

A series of key parameters need to be considered when designing an algal growth system. These parameters include gas exchange efficiency (especially for carbon (CO_2) supply and oxygen concentration) (Kuentzel, 1969; Kumar et al., 2010; Zhao & Su, 2014), illumination (light intensity and distribution) (Carvalho et al., 2011; Grima et al., 1999; Wijffels & Barbosa, 2010) and nutrients supply (Béchet et al., 2013; Chen et al., 2011b; Yang et al., 2011). A trade-off is needed when considering all the key parameters to ensure a good growth system instead of meeting all the requirements.

Table 2.1: Biomass yield rate, oil yield rate, and methane potential of some typical studied algal species.

Microalga	Biomass yield rate ($t\ ha^{-1}\ y^{-1}$)	Lipid content (% dry weight)	Methane potential ($L\ CH_4\ Kg\ VS^{-1}$)	Ref.
Arthrospira (Spirulina)	33	13 %		(Torzillo et al., 1986)
Botryococcus braunii	18-33	25-75 %		(Chisti, 2007)
Chlorella sp.	25-50	28-46 %	230-580	(Moore, 2001; Stephenson et al., 2010; van Beilen, 2010)
Dunaliella salina	60	23-30 %		(Hosseini Tafreshi & Shariati, 2009)
Nannochloropsis sp.			100-300	(Alzate et al., 2014)
Tetraselmis sp.	45	25-42 %	200-400	(Bohutskyi et al., 2014; Marzano et al., 1982)

NA: not available.

Carbon limitation

Carbon limitation has been confirmed in both indoor and outdoor experiments (Azov et al., 1982; Goldman & Graham, 1981). Kuentzel (1969) revealed that the large amounts of CO_2 required for mass cultivation of algae cannot come from the atmosphere alone (with CO_2 levels at ~0.0387% (v/v)) and/or dissolved carbonate salts via the normal physical-chemical processes. At most, about 1 mg L^{-1} of free CO_2 accumulated over a period of many hours to days can be expected from the atmosphere via mass transfer at room temperature. From the reported algal species, CO_2 addition (within a certain range) to algal culture has been demonstrated to achieve higher algal photosynthetic efficiencies and productivities compared to controls without CO_2 addition (Yang, 2011; Zhao & Su, 2014). Cheng *et al.* (2006) concluded that maximum algal productivity was obtained when the concentration of CO_2 in the aeration gas was 1.0% in an experimental photobioreactor. Azov and Goldman (1982) found that CO_2 addition to a pilot-scale high rate algal pond (HRAP) more than doubled algal production compared to the one without CO_2 addition. Section 2.3.1 outlines the potential avenues to overcome

carbon limitation, which provides the neseccery background information needed for the Research Objective 1.

Oxygen inhibition

Oxygen at high concentration in the cultures would inhibit severely the growth of algae (Peng et al., 2013; Pulz, 2001). Therefore effective de-oxygenation is required to maintain dissolved oxygen at a level that is not inhibitory. Different algal species have very different tolerance to O_2, ranging from 7.67 mg O_2 L^{-1} (at air saturation at 30 °C) (Sánchez Mirón et al., 1999) to 15.34 mg O_2 L^{-1} (at 200 % of air saturation at 30 °C) (Weissman et al., 1988). Keymer *et al.* (2013c) revealed that oxygen production, a surrogate for algal activity, is inhibited at elevated DO and inhibition can be described by a Hill model. Oxygen inhibition can be caused by dissipation of light energy through photorespiration, inhibiting photosynthetic enzymes, damaging to the photosynthetic apparatus (especially photosystem II, the first protein complex which accepts electron from oxygen) and other cellular components (Ma & Gao, 2010; Santabarbara et al., 2002; Ugwu et al., 2007).

Nutrient limitation

The requirement for nutrients supplementation is another bottleneck in the algal mass cultivation process. Similar to terrestrial plants, algae require several inorganic nutrients for full-speed growth, especially nitrogen and phosphorous (Richmond, 2004). Recycling of nutrients from the other processes in the algal biofuel pipeline would make the whole process more feasible (Rösch et al., 2012). Anaerobic digestion (Baisuo et al., 2009; Keymer et al., 2013b) and hydrothermal gasification (Haiduc et al., 2009) are the most investigated pathways for nutrients cycling. However, the technologies are either still at an experimental stage (hydrothermal gasification) or are applied at industrial scale but not yet with algae (anaerobic digestion).

2.2.2 Harvesting

After the growth of algae, algal biomass needs to be harvested/concentrated from the culture broth (normally with a solid concentration less than 0.2 %), and then be processed to release the products, such as lipids to produce biodiesel (Schenk et al., 2008), or be fed to anaerobic digestion to produce methane (as shown in Figure 2.2). Harvesting/concentrating algal biomass can be energy intensive and it alone accounts for about 20 %-30% of the entire algal biofuel pipeline cost (Rawat et al., 2011). Therefore, efficient harvesting is essential to reduce the process cost. Available harvesting options are outlined below.

- Centrifugation is conducted by gravitational force to separate algal culture broth into pellet and supernatant. It has been proved to be effective in harvesting algae with recovery in excess of 90 % by centrifugation. However, the high energy requirement of centrifugation makes it only feasible for laboratory tests and secondary thickening of harvested algae (1-2 %) (Alabi et al., 2009; Park et al., 2011) and for large algae (ca. >70 μm) (Alabi et al., 2009).

- Sedimentation is conducted with gravitational forces causing liquid or solid particles to separate from a liquid of different density. Sedimentation of colonial and larger algae could be useful as a pre-concentration process before other harvesting methods. The major drawback of sedimentation is that the process can be extremely slow since the density difference of algae (1.03 – 1.13 g L^{-1}) and water (1.00 g L^{-1}) is too small and that the particle size is too small (generally algal cell diameter is 5 μm to 20 μm) (Milledge & Heaven, 2013).

- Flotation is conducted by dissolved air which involves pressurizing some of the liquid to dissolve additional air bubbles (Singh et al., 2011). When the pressurized liquid is mixed with the algae culture at atmospheric pressure, the air comes out of solution as bubbles that attach to the particles, making them float. Flotation processes can be classified according to how the air bubbles are produced: dissolved air flotation (DAF), electrolytic flotation (EF), and dispersed air flotation (Milledge & Heaven, 2013).

- Filtration is a harvesting technique according to size difference of particles. By introducing the particles onto a filter/membrane of a given pore size, the particles can then be separated according to their sizes. Pressure or vacuum is often applied to enhance the filtration process. A major issue of filtration is filter/membrane clogging and fouling which increases the entire

filtration costs (Christenson & Sims, 2011; Milledge & Heaven, 2013).

Flocculation occurs when the solute particles collide and adhere to each other. Flocculation can occur naturally in certain algae or a mixed algae culture (Salim et al., 2010; Spilling et al., 2010; Sukenik & Shelef, 1984), which is called auto-flocculation. Flocculants can be added to the culture broth to enhance the flocculation efficiency and therefore increase the settling rate (Kim et al., 2011; Lee et al., 1998; Pushparaj et al., 1993). There are two main types of flocculants used to induce flocculation: inorganic flocculants and organic polymer/polyelectrolyte flocculants. Alternatively, instead of conventionally introducing bio-flocculants into algae culture for harvesting, the use of microalgal bacterial flocs (MaB-floc) has been proved feasible for some algal and bacterial species (Van Den Hende et al., 2011a; Van Den Hende et al., 2011b).

Most algal harvest and recovery techniques discussed above have been developed based on technologies that have been used in the water purification industry (Kim et al., 2013). Although there are technical similarities between algal harvest and water purification, it is necessary to develop approaches that can address the technical needs that are unique to harvesting algae. The new approaches must consider the following aspects: 1) The approaches should be algal species agnostic; 2) the technologies must have a strong potential to be integrated into existing upstream and downstream steps; and 3) the technologies must be scalable to meet the challenge of commercial bioenergy or bioproducts production.

2.2.3 Pre-treatment

Pre-treatment of algal biomass is usually required prior to downstream processing, ie. lipid extraction and anaerobic digestion (Figure 2.2). The high energy consumption for lipid extraction is because when solvent-based extraction techniques are applied to a wet algal paste after harvesting, the cells tend to remain intact due to their cell envelope (cell wall and membranes), which prevents them from getting direct contact with the solvent. Similarly, the practical methane potential of algae is often much lower than the theoretical value, again due to the cell envelope, which prevents microorganisms getting access to the intracellular components. The algal cell envelope is a thick and rigid layer consisting of complex carbohydrates and glycoproteins with high mechanical strength and chemical resistance (Kim et al., 2013). So far, various pre-treatment methods have been investigated, including microwave, sonication, bead beating, autoclave, thermal hydrolysis, osmotic shock, homogenization, and grinding, *etc*. This section provides a general introduction on the current pre-treatment

technologies and identifies the bottlenecks. Section 2.4 goes into details of current pre-treatment technologies and potential novel method. Section 2.4 which provides the neseccery background information needed for the Part II of the thesis (Research Objectives 2 and 3).

Generally, the pre-treatment methods are classified into three types: mechanical, chemical, and biological.

There are diverse mechanical methods, such as microwave, sonication, bead beating, high pressure thermo-hydrolysis, and electroporation, etc. Mechanical pre-treatment methods generally directly break cells through physical forces, which has been proved relatively efficient for cell disruption. The biggest advantage of which is that these methods can be universally applied to algal biomass regardless of its species. However, the major drawback of using mechanical methods for algae pre-treatment is the intensive energy consumption (Kim et al., 2013).

Chemical methods are also studied by researchers, such as treatment with acids (Miranda et al., 2012), alkaline, surfactants, or heated chemicals (Sarada et al., 2006) which can degrade chemical linkages on the rigid cell envelope. Compared to mechanical methods, chemical methods are less energy intensive; yet their pre-treatment efficiency is relatively low or species dependent.

Biological methods refer to the methods that degrade the algal cell envelope using enzymes or other biological reagents (Young et al., 2011; Zheng et al., 2011). Compared to the mechanical methods which destroy almost every particle existing in the solution, the biggest advantages of using enzymatic methods are the mild reaction conditions and the high selectivity. However, the critical downfall of the biological method is the high cost of the enzymes, and the potential to scale-up these methods is still limited since they require continuous use of expensive chemicals and enzymes.

As discussed above, each process offers several advantages and disadvantages, and more research work still need to be done to enhance the harvesting process efficiency, meanwhile decrease the process costs.

2.2.4 Lipid extraction

Lipid extraction from algae is usually conducted by solvent extraction, according to the ‘like dissolving like’ concept. Similar to commercialized solvent extraction from oil plants, the most used organic solvents are n-hexane, chloroform, ethanol, and mixed solvents such as hexane/isopropanol,

hexane/ethanol, chloroform/methanol (Fajardo et al., 2007; Halim et al., 2012; Halim et al., 2011). The criteria of organic solvent selection are extraction efficiency, cost for distillation from the crude lipids and operation friendly (low toxicity).

Additionally, several green extraction techniques (especially on compounds with pharmaceutical importance, such as carotenoids, and Chlorophyll a) have been investigated (Macías-Sánchez et al., 2007; Mendes et al., 2003). For example, supercritical carbon dioxide extraction ($SCCO_2$) uses $SCCO_2$ fluid to provide a solvent power. It can be continuously adjusted by changing the extraction pressure and temperature, in which way the solvent power of $SCCO_2$ tunes such that it interacts primarily with neutral lipids (Macías-Sánchez et al., 2007).

2.2.5 Biodiesel production

Transesterification, also called alcoholysis, is the standard industrial technology to convert extracted triacylglycerols (TAG) from algal cells into biodiesel. It has already been commercially used to generate biodiesel from other oils, especially plant oils (Felizardo et al., 2006; Warabi et al., 2004). The basic principle of this process is the reaction of triacylglycerides (microalgal oil) with an alcohol (usually methanol) in the presence of a catalyst to produce methyl esters (microalgal biodiesel) and glycerol which is shown in (Figure 2.3) modified from Chhetri and Islam (2008). Among all the options, methanol is most commonly used since it is the cheapest alcohol available. Using higher molecular weight alcohols improves the cold flow properties of biodiesel but reduces the efficiency of the transesterification process (Xia et al., 2009). Four catalyzed processes have been investigated including alkali-, acid-, lipase-, and supercritical catalysis (Haas, 2005; Kim et al., 2004; Shieh et al., 2003). The alkali-catalyzed process is almost three times faster than the acid-catalyzed process and is much cheaper than the lipase-catalyst, which makes alkali-catalyst broadly used (Fukuda et al., 2001).

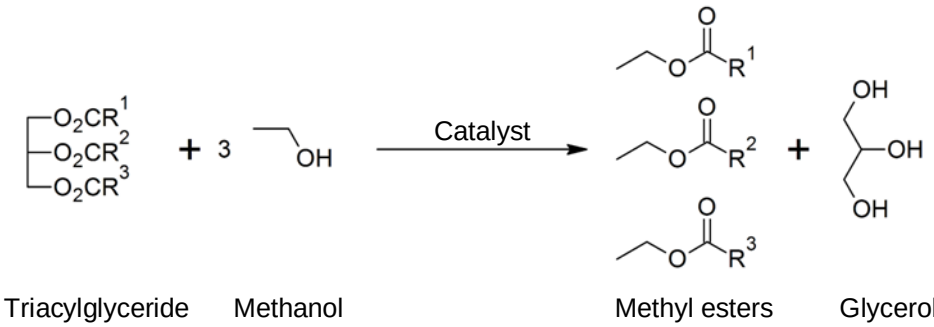


Figure 2.3 Transesterification of triacylglycerides to methyl esters (biodiesel). R^{1-3} represent hydrocarbon groups. Figure modified from (Chhetri & Islam, 2008).

Problems of solvent lipid extraction can be identified as i) low extraction efficiency, ii) high costs , especially cost for distillation from the crude lipids and iii) hazardous operation when using organic solvents, such as chloroform.

Transesterification has been commercially implemented for the conversion of waste plant oils, and that whole production process can be easily used to convert algal lipids, as they are essentially the same feedstock (Chhetri & Islam, 2008).

2.2.6 Anaerobic digestion and biomethane production

Anaerobic digestion (AD) of algae has potential to yield up to 800 L CH_4 Kg VS^{-1} (Heaven et al., 2011). AD is a more straightforward process to produce energy, and does not require intense concentration, drying or oil extraction. However in practice, the methane yield of algae from AD is much lower than the theoretical methane yield (Richmond, 2004). A couple of factors may contribute to the lower than excepted methane yield, including the physical characteristics of algae (cell envelope and cell characteristics, *etc.*) (Passos et al., 2014; Passos et al., 2013) as well as the inhibitory effects of both substrate and by-products (such as ammonia) (Chen et al., 2008; Ras et al., 2011).

The cell envelope (cell wall and membrane) of algae has been described as a rigid barrier which would influence the accessibility of anaerobic microorganisms to the substrates, and thereby determine the methanogenic conversion efficiency. The rigid cell envelope of algal cells is one of the major reasons for the relatively low methane yield compared to the theoretical value. The low carbon/nitrogen (C/N) ratio of algal biomass is also responsible for the relatively low methane yield. The C/N ratio is an important factor for guaranteeing the stability of the anaerobic digestion process (González-Fernández et al., 2012; Yen et al., 2013). Other limitations of anaerobic digestion on algal biomass are the same as anaerobic digestion on other feedstocks, ie. the operating conditions, such as temperature, hydraulic retention time and loading time (Sialve et al., 2009).

Additionally, inhibition factors induced by algal feedstock production process need to be considered when designing the entire process. Researchers are arguing that digestion the post-lipid extraction algal residues is a necessary step to guarantee the sustainability of algal biofuel production. However, some solvent residues in algal biomass after lipid extraction have turned to be detrimental to anaerobic

digestion. Baisuo *et al.* (2009) reported that chloroform residue can totally inhibit the methanogenesis from anaerobic digestion on post-lipid extraction algal residues. Other factors can also significantly inhibit AD, including ammonium toxicity, sodium toxicity, and nitrite toxicity, *etc.* (Banihani et al., 2009; Sialve et al., 2009).

Section 2.4.2 outlines a potential novel method for methane yield enhancement, which provides the necessary background information needed for the Research Objectives 3.

2.3 Open algal culture systems (Part I)

The first part of the thesis focuses on algal growth in open culture systems. Two subtopics are considered, i) enhancing algal growth by bacterial carbon cycling in open culture systems and ii) developing an Algae-Bacteria Model for open algal culture systems.

Model based process control of algae production has been considered by some researchers and engineers (Baquerisse et al., 1999; Béchet et al., 2013; Goldman et al., 1974). But only a handful of models on biomass production in open algae cultivation system are currently available, especially considering the activity of heterotrophic bacteria (Buhr & Miller, 1983; Fallowfield & Garrett, 1985; Jupsin et al., 2003; Mesplé et al., 1996). This is in comparison with more 40 years of model development in the related field of biological wastewater treatment. The work in that field has resulted in models such as the activated sludge models (ASM series) (Henze et al., 2000), which accurately describe the dynamics of carbon and nutrient removal in the activated sludge processes.

Modelling algal activity in open systems is challenging because (i) biological processes interact with the organic and inorganic carbon levels (*e.g.* autotrophic behaviour of algae and heterotrophic behaviour of bacteria) (Banga et al., 2010), and (ii) complex physical limitations (*e.g.* light attenuation and distribution) (Béchet et al., 2013; Bernard & Rémond, 2012). The following sections review the former (namely algae bacteria interactions) as well as the opportunity to develop a new model for algae production based on the successful format of the ASM series.

2.3.1 Carbon limitation

As shown in the following mass transfer equation, carbon is generally dissolved into the culture broth by mass transfer of inorganic carbon (CO_2).

$$r = K_{LaCO2}(S^*_{CO2} - S_{CO2}) \qquad \text{Equation 2.1}$$

where K_{LaCO2} (d^{-1}) is the mass transfer efficiency, S^*_{CO2} (mg C L^{-1}) is the saturated dissolved CO_2 concentration, and S_{CO2} (mg C L^{-1}) is the dissolved CO_2 concentration.

Systems that rely on conventional carbon supply strategies (e.g. air bubbling, paddling etc.) are carbon limited due to i) low transfer efficiency (low K_{LaCO2}), which is a function of the aeration infrastructure and reactor configurations as well as the high surface tension of water, and ii) low driving force due to low S^*_{CO2}, which is a consequence of the relatively low CO_2 content of the

atmosphere (0.038% v v^{-1}) (Sander, 1999).

The need for additional CO_2 for mass algal biomass production (and the requirements of CO_2 for algal blooms) has long been known (Kuentzel, 1969; Kumar et al., 2010). As shown in Figure 2.4, many reported algal species are suffering from carbon limitation if supplementation of CO_2 comes from atmosphere alone (with CO_2 concentration at 0.038 (%, v/v)). CO_2 addition (within a certain range) to an algae culture can help achieve higher algal photosynthetic efficiencies and productivities compared to controls without CO_2 addition (Table 2.2). The optimal CO_2 concentration (as concentration in the supplied gas) for most algal species is usually recommended to be 2-15 %, e.g., the maximum biomass production was observed at around 5 % CO_2 (%, v/v) for algae *Chlorella* sp T-1 (CS T-1) (Maeda et al., 1995) while at around 12 % CO_2 (%, v/v) for *Scenedesmus obliquus (*SO) (de Morais & Costa, 2007).The main strategy to boost the driving force is to use CO_2 enriched gases (such as flue gas). This has been demonstrated at both laboratory scale and pilot scale, where CO_2 addition (up to a point) has led to higher algal photosynthetic efficiencies and productivities compared to controls (Yang, 2011; Zhao & Su, 2014). Azov *et al.* (1982) found that algal production in a pilot-scale high rate algal pond (HRAP) with CO_2 addition was more than double that achieved in one without CO_2 addition.

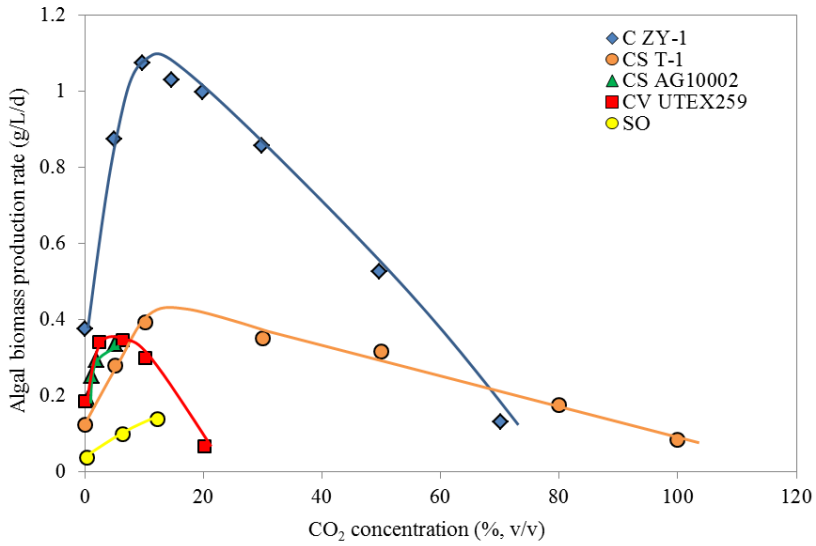


Figure 2.4 Effect of CO_2 concentration on algal biomass production. C ZY-1=*Chlorella* ZY-1 (Yue & Chen, 2005), CS T1=*Chlorella* sp. T1 (Maeda et al., 1995), CS AG 10002=*Chlorella* sp. AG10002 (Ryu et al., 2009), CV UTEX259=*Chlorella vulgaris* UTEX259 (Yun et al., 1996), SO=*Scenedesmus obliquus* (de Morais & Costa, 2007).

Table 2.2: Summary of algal biomass production and CO_2 fixation parameters under different process conditions.

Algal species	**CO2 concentration**	**Aeration rate**	**Initial density**	**Light intensity**	**L/D**	**pH**	**Operating strategy**	**μ**	**Biomass production rate**	**Carbon fixation rate**	**Ref.**
	(%, v/v)	**(vvm)**	**(g/L)**	**(μmol/m2/s)**	**(h/h)**			**(1/d)**	**(g/L/d)**	**(g/L/d)**	
C ZY-1	0.038-70	0.25	0.1	140	12/12	7.0-3.0	Batch	0.347-0.667	0.117-0.950	0.219-1.768	(Yue & Chen, 2005)
CS T-1	0.038-100	0.83	0.002-0.005	110	NA	NA	Batch	0.542-0.840	0.084-0.394	0.158-0.741	(Maeda et al., 1995)
CS AG10002	0.5-5	0.1	0.01	100	24/0	7.2 (initial)	Batch	0.792-0.885	0.192-0.335	0.360-0.630	(Ryu et al., 2009)
CV UTEX259	0.038-20	2	0.1	110	24/0	7.2-5.4	Batch	0.216-0.385	0.067-0.344	0.063-0.648	(Yun et al., 1996)
SO	0.038-12	0.3	0.15	44.8	12/12	7.4-5.3	Batch	0.216-0.261	0.064-0.090	0.120-0.169	(de Morais & Costa, 2007)

2.3.2 Interactions between algae and bacteria

In both natural and engineered systems, algae and bacteria are found together in loose or tight association. Bacteria and algae are the most numerically dominant organisms in oceans and lakes. In both fresh water and marine systems bacteria usually number between 10^4 to 10^6 cells per ml (Bell, 1982). Marine bacteria and algae have complex interactions leading to parasitism, mutualistic symbiosis and co-existence (commensalism) (Cole, 1982). Biologists are trying to understand the mechanisms of interactions between bacteria and algae in natural ecosystems, which not only can give a better prediction on global changes in primary production, nutrient and carbon cycling, but also can help researchers obtain a better understanding on how these algae can be successfully grown in culture for further applications.

It is well documented that numerous interactions occur in algal-bacterial systems (Cole, 1982). It is generally thought that the algae and bacteria maintain symbiotic relations. Several reports indicate that algae grown in the presence of bacteria typically grow better than under axenic (pure cultures of microorganisms that are not contaminated by or associated with any other living organisms) conditions (Andersen, 2005; Fukami et al., 1997; Riquelme et al., 1987; Suminto & Hirayama, 1997).

The stimulative effect of bacteria on algal growth has been described as being associated with bacteria mineralising carbon (Ask et al., 2009; Muñoz & Guieysse, 2006), as well as cycling nutrients (Bloesch et al., 1977; Zhao et al., 2012) and generating vitamins (Grant et al., 2014)and other growth promoting regulators. Considering inorganic carbon limitation, this work focuses on bacterial stimulation via mineralising photosynthetic end products.

Considering bacteria mineralising carbon, this carbon cycling process has potential to help overcome carbon limitation in open algal culture systems. The most widely investigated application of bacteria carbon cycling is in the realm of bioremediation. Algae are useful microorganisms in bioremediation applications. Use of algae as a means of wastewater treatment was pioneered by Oswald and his co-workers in the late 1960s (Oswald et al., 1957; Oswald, 1962; Oswald & Gotaas, 1957). As shown in Figure 2.5, the use of algal-bacterial consortia during the tertiary bio-treatment of wastewater reduces operation costs in situations when oxygen must be provided externally and mechanically because algae can provide the O_2 required by heterotrophic bacteria to degrade organic pollutants (Muñoz & Guieysse, 2006), meanwhile a significant cost reduction can be achieved by the use of waste media as it negates the need to purchase growth media (Medina & Neis, 2007).

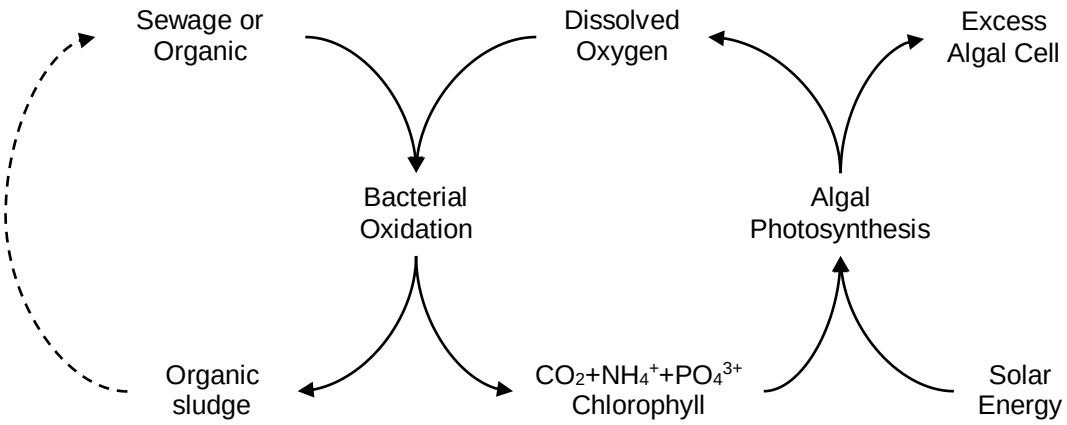


Figure 2.5 Principle of bacterial carbon cycling during bioremediation by algal-bacterial consortia.

2.3.3 Carbon cycling by bacteria

As shown in Figure 2.5, carbon flow is one of the most important material flows in open algal culture systems. Complementary to conventional carbon supply strategies, carbon cycling should be considered. This strategy could help overcome carbon limitation in open algal culture systems. Carbon cycling refers to the oxidation of the products of photosynthesis to CO_2, which is then available to be re-processed into organic compounds by further photosynthesis. In open algal culture systems, oxidation is driven by heterotrophic bacteria.

Mineralisation of organic carbon underpins the design of some algae-based wastewater treatment systems. Some mathematical models of these systems, including high rate algal-bacterial ponds, consider links between algae and bacteria (Buhr & Miller, 1983; Jupsin et al., 2003) with Yang (2011) even focusing on CO_2 supply and utilization in these systems. Yang showed that bacterial driven carbon oxidation, albeit oxidation of supplemented organic carbon, can play an important role in supporting algal activity. However, neither Yang (2011) nor the other authors, have quantified the contribution of bacterial carbon cycling (that is oxidation of products of photosynthesis) to algal productivity.

Research objective 1 covers the understanding of bacterial carbon cycling to open algal culture systems. The objective of this study was to understand the contribution of bacterial carbon cycling to algae growth both by experiments and modelling. Since the pH level largely controls the proportion of inorganic carbon pools in the liquid phase, two different pH values for the batch test were examined

to validate the model. Modeling was developed to aid illustrating the complex carbon flow in an open algal culture system.

2.3.4 Algae-based models

Several algal growth models have been developed by researchers and engineers since the 1960s. Considering the effect of light intensity on specific growth rate of algae, three basic algal growth models have been developed,

i) Monod equation if no photoinhibition is observed (Grima et al., 1994; Holmberg, 1982),

ii) Haldane equation (Equation 2.2) when photoinhibition occurs (Chisti, 2007)

iii) Steel equations (Equation 2.3) when photoinhibition and cell self-shading are considered (Baquerisse et al., 1999; Benson et al., 2007).

$$\mu = \mu_{max} \frac{I}{I+K_I} \quad \text{Equation 2.2}$$

$$\mu = \mu_{max} \frac{I}{I+K_I+\frac{I^2}{K_2}} \quad \text{Equation 2.3}$$

where μ_{max} is the maximum specific growth rate of algae, I is light intensity, K_I is the saturation constant for light intensity.

Based on the algal growth models described above, researchers are trying to build more comprehensive models to better predicting algal growth under different circumstances. In the River Water Quantity Model No.1 (RWQM1) (Reichert, 2001; Reichert & Borchardt, 2001; Reichert et al., 2001a; Reichert et al., 2001b; Reichert et al., 2001c; Reichert & Vanrolleghem, 2001), autotrophic algal growth was characterized by a Monod model divided into two sub-processes describing growth with ammonia or nitrate as the nitrogen source, and a Steel function to describe light limitation and light inhibition (Reichert et al., 2001b).

The river water model includes major processes and appropriate kinetic and stoichiometric expressions for wastewater treatment systems and river water ecosystems, taking into account algae, bacteria and other microorganisms coexist; leading to complex models describing all the major processes of bacteria, algae, and grazers. However, although bacterial processes were described in detail, algal processes were simplified in many aspects, which makes the models not suitable to describe algal cultivation for biofuel and other by-products production. Thus, an algal-based model

describing the algal carbon assimilation kinetics with consideration of the presence of bacteria is required. Such a model is developed as part of Research Objective 1 in this thesis.

2.4 Downstream process enhancement by pre-treatment (Part II)

The second part of the thesis focuses on the downstream process enhancement for biofuel production, including lipids recovery for biodiesel production and methane production from anaerobic digestion process. The following sections provide the necessary background required for the Part II of the thesis (Research Objectives (RO2 and 3)).

2.4.1 Lipid extraction and biodiesel production

Regarding to lipid extraction for biodiesel production, solvent extraction is one of the most widely applied methods to recover lipids, but a great challenge of this process is difficulty in accessing intracellular lipids. This is identified as a major bottle-neck for large-scale production of algal biodiesel. The main reason is that algae possess a cell envelope (cell wall and cell membrane), which is a thick and rigid layer consisting of complex carbohydrates and glycoproteins with high mechanical strength and chemical resistance (Kim et al., 2013).

In order to overcome the limitation in lipids recovery from algal biomass, several pre-treatment techniques have been investigated as introduced in Section 2.2.3. Chemical or biological cell disruption techniques are attracting escalating attention to improve lipid extraction yield or efficiency (Jin et al., 2012; Lee et al., 2010; Mendes-Pinto et al., 2001). Despite the high cell-disruption performance of the chemical and biological treatments, the potential to scale-up these methods is still limited since they require continuous use of expensive chemicals and enzymes. Therefore, alternative powerful pre-treatment technique is needed.

Recent studies showed that free nitrous acid (FNA or HNO_2), which is the protonated species of nitrite, has strong cellular destruction and enzyme interference effects on several microorganisms (Jiang et al., 2011). FNA has been regarded as a biochemical reagent since several works also revealed that FNA and its derivatives such as nitric oxide ($NO\cdot$) and nitrous anhydride (N_2O_3) have effect on protein and polysaccharides degradation (Dedon & Tannenbaum, 2004). Hence FNA has been applied in the water and wastewater industry for sludge treatment, biofilm control etc. Since FNA combines chemical effect and biological effect to algal cells, it is expected that FNA pre-treatment could help disrupt the algal cell envelope barrier, thereby increasing the rate of lipid mass transfer from the algal cells into the organic solvent.

Research Objective 2 then focus on boosting lipids and specifically TAG, recovery from algal by

applying FNA pre-treatment.

2.4.2 Anaerobic digestion and biomethane production

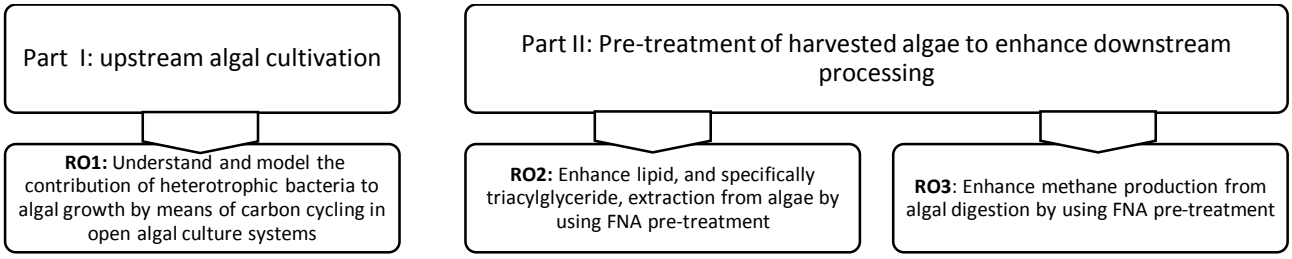
As described in Section 2.2.6, a number of algae pre-treatment techniques which can potentially increase methane yields have been investigated, including thermal hydrolysis, ultrasound, microwave, chemical and enzyme hydrolysis methods, etc. (Cho et al., 2013; Keymer et al., 2013b; Mahdy et al.; Passos et al., 2014). These technologies destroy cells and release intracellular and/or extracellular constituents to the liquor phase. The disrupted cells and released constitutes are more readily biodegraded during AD, therefore enhancing methane production yield. However, most of the above pre-treatment techniques are cost intensive due to high energy and/or chemical requirements (Singh & Olsen, 2011). Thus, alternative pre-treatment techniques to enhance methane production from AD are needed.

A potential alternate pre-treatment technique that has already been demonstrated to be effective for improving digestibility of waste activated sludge (WAS) from wastewater treatment plants (WWTP) is FNA pre-treatment (Wang et al., 2013). When digesting WAS from wastewater treatment plants, FNA pre-treatment (at 1.78-2.13 mg HNO_2-N L^{-1}) enables an improvement on methane potential, with the highest improvement being approximately 27% (from 201 to 255 L CH_4/Kg VS added) compared to untreated WAS (Wang et al., 2013).

Therefore, Research Objective 3 focuses on the biogas production from algae by investigating the feasibility of using FNA pre-treatment technique to boost methane production.

3 RESEARCH OBJECTIVES

The thesis is divided in two parts: (i) upstream algal cultivation, and (ii) pre-treatment of harvested algae to help downstream bioenergy production. .



3.1.1 Research Objective 1 (RO1) – Understand and model the contribution of bacteria to algal growth by carbon cycling in lab-scale algal culture system

Algal mass production in open systems is often limited by the availability of inorganic carbon substrate. Heterotrophic bacteria have the potential to help overcome the carbon limitation by recycling inorganic carbon to algae. This research objective is to evaluate how heterotrophic bacterial driven carbon cycling mitigates carbon limitation in open algal culture systems both by experiments and modelling. A kinetic model is proposed which will allow description of the complex carbon flux in open algal culture systems, considering the present of heterotrophic bacteria.

Until now, only few models on algal mass cultivation, considering the activity of bacteria, have been published. An extension of this objective is to develop an integrated model that describes autotrophic and heterotrophic growth in open algal cultures, together with the major physico-chemical processes. This model will provide a description of algal cultivation processes considering the effect of heterotrophic bacteria driven carbon cycling and oxygen consumption.

Hypothesis:

The carbon limitation for algal growth in open culture systems will be overcome by the heterotrophic bacterial activity to re-mineralise photosynthetic end products. A kinetic model will allow further understanding on the carbon cycling driven by heterotrophic bacteria under varying pH values.

3.1.2 Research objective 2 (RO2) – Enhance lipid, and specifically triacylglyceride (TAG), extraction from algae by using free nitrous acid pre-treatment

Lipid extraction has been identified as a major bottleneck for large-scale algal biodiesel production. The main barrier of lipid extraction using solvents is the rigid cell envelope (cell wall and cell membranes) posed by algal cells. Effectively disrupting the cell envelope will allow enhanced lipid extraction from algae.

Of the lipids extracted it is the triacylglyceride (TAG) content that matters for biodiesel production since TAG are the feedstock which can react with methanol and then produce fatty acid methyl esters (FAME). This research objective is to develop an alternative pre-treatment method - free nitrous acid (FNA) pre-treatment – and investigate its ability to boost total lipid and TAG recovery from algae for biodiesel production.

Hypothesis:

The algal cell envelope will be disrupted by FNA pre-treatment, which will lead to enhanced lipid and consequently TAG recovery. There is also potential to improve the extraction rate by increasing the mass transfer rate of lipid into organic solvents, which is particular important for large scale application. However, consideration is required to determine an optimum FNA concentration for TAG fatty acids recovery, since high concentration FNA is potentially detrimental to some kinds of fatty acids.

3.1.3 Research objective 3 (RO3) – Enhance methane production from algal digestion by free nitrous acid pre-treatment

Anaerobic digestion (AD) of algal biomass is a necessary step to make the algal biofuel production feasible. However methane yields for algal digestion are often much lower than this. A couple of factors may contribute to reduced yields, including the physical characteristics of algae as well as the inhibitory effects of both substrate and by-products (such as ammonia). In particular, the cell envelope of algae has been described as a rigid barrier and therefore limits access for biological activity. This research objective aims at developing an alternative pre-treatment technology by using free nitrous acid (FNA) to improve methane production.

Hypothesis:

FNA pre-treatment has the ability to help cell disruption and components solubilisation, which will increase the methane yield from algal biomass. Also, the FNA remained has potential detrimental influence on the next step processing.

4 REASERCH APPROACH

The research approaches that underpinned this thesis are outlined as follows. Section 4.1.1 details the source of algae and bacteria. Section 4.1.2 outlines the general growth and culture conditions of algae. Section 4.1.3 provides the research approach for investigating Part I research objectives and Sections 4.1.4-4.1.6 for Part II research objectives. Section 4.1.7 summarises the research method for revealing how FNA pre-treatment disrupt cells.

4.1.1 Algae and bacteria source

Two algal sources were used in this thesis. Fresh water algae *Chlorella* sp. was applied for Research Objective 1 due to its sole affiliation to free carbon dioxide during growth. This algae was isolated from UQ lake (The University of Queensland, Australia). Marine algae *Tetraselmis striata* M8 was used for the Research Objective 2 and 3 since it has been proven useful for large scale application due to its high productivity and lipid content. This algae was collected in an intertidal rock pool at Maroochydore (Sunshine Coast, Queensland, Australia).

Heterotrophic bacteria inoculum for Research Objective 1 was obtained from a sequencing batch reactor treating synthetic domestic wastewater, maintained at The University of Queensland, Australia.

The inoculum for Research Objective 3 was harvested from a mesophilic anaerobic digester treating mixed primary sludge in the WWTP (Queensland, Australia). The reason for using bacteria inoculum from sequencing batch reactor treating synthetic wastewater is that it is conventional to consider biomass from WWTPs to be generic heterotrophic cultures.

4.1.2 Algal growth and culture conditions

Two main growth systems were applied to grow algae for the experiments in this thesis. One is a set of bench-top laboratory open reactors and the other one is an outdoor conical open-top bioreactor for larger scale biomass accumulation. The laboratory reactors were used to investigate and quantify the contribution of bacteria to algal growth through carbon cycling and to verify the application of the ABM on carbon dioxide and oxygen changes (Research Objective 1); while the outdoor open algal bioreactor was used to accumulate larger volumes of algal biomass for FNA pre-treatment assays and bioenergy production (Research Objective 2 and 3).

For Research Objective 1, *Chlorella* sp. was pre-cultured in 3 L airlift photobioreactors for three months and acclimatized by feeding f/2 media (Varicon Aqua, Worcestershire, UK). The 3 L airlift photobioreactors are 80 cm × 15 cm (Height × DI) cylindrical reactors. As for the trials, open algal culture systems were conducted. Cylindrical open reactors were used for the batch tests of Research Objective 1. The specification of the cylindrical open reactors is 20 cm × 15 cm (Height × DI). pH of the growth media was buffered by 20 mM HEPES (Sigma-Aldrich Pty. Ltd) and maintained as constant through cultivation. HEPES is an organic zwitterionic buffer commonly used in algae growth studies. The pH was monitored with a portable pH probe. Air bubbling was supplied at a constant speed ($K_{La_{O2}}$=36 d^{-1}). Light was generated by a 36 W fluorescent lamp (Osram, 18 W/840) from one side of the reactors. Lighting was operated on a 12 h light, 12 h dark period, and the light intensity was measured to be 180 µmo photons m^{-2} s^{-1} at the incident side of the reactors by a light meter (840020C, Sper Scientific Ltd., USA). The experiment was operated in a temperature-controlled room with constant ambient temperature at 23 ± 0.2 ˚C.

For Research Objective 2 and 3, *Tetraselmis striata* M8, which is a marine algae with high lipid accumulation ability, was cultured in 40 L airlift photobioreactors at the University of Queensland. f/2 medium was used as the growth media. The 40 L airlift photoreactors are clear 20 litlyophilizre polyethylene bags with an average thickness of 15 cm. Culture pH was kept constant at 8.5±0.2 by CO_2 injection with an electronic controller, and the depletion of nutrients ($NO_3^-$and PO_4^{3-}) was tested using seawater aquaria nutrients kits (DAPI Aquarium Pharmaceuticals for NO_3^- and Nutrafin for PO_4^{3-}). Nitrogen starvation strategy was used to accumulate lipids in the algal cells. After 5 days cultivation for algae growth and 3 days for lipid accumulation. The algae was grown to a dry weight of around 1 g L^{-1} and then 300 L algal culture was concentrated to a paste by centrifugation at 3270 g (Beckman Coulter, Allegra™ X-12) for 3 min in 6 L batches. The algae paste was collected for FNA pre-treatment (described in Section 4.1.4).

4.1.3 Lab-scale reactors operation for Research Objective 1

i. Batch tests for understanding the contribution of bacteria to algal growth

Two sets of lab-scale open algal growth reactors, carried out at two different pH values, were conducted to examine the effect of the bacteria supplementation. f/2 media was added to the reactors to supply sufficient nutrients for algal growth. Nutrients for algal growth were monitored by off-line analysis to ensure that they were in excess. Batch 1 was operated at pH 8.5 in triplicate for 7 days.

Six reactors were run in parallel with two different initial bacteria to algae ratios (1.1 and 9.2 respectively on dry mass basis). Batch 2 was operated at pH 7.5 in duplicate and cultivation time was extended to 9 days. Four reactors were run with two different initial bacteria to algae ratios (1.1 and 9.0 respectively on dry mass basis). Detailed experimental conditions of two batch experiments are shown in Equation 4.1. The growth media was buffered by 20 mM HEPES at 8.5±0.2 for Batch 1 and 7.5±0.2 for Batch 2. The pH was monitored with a portable pH probe. All of the bench-scale growth reactors were 330 ml glass reactors, well mixed by magnetic stirrers at a constant speed of 350 rpm. Light was generated by a 36 W fluorescent lamp (Osram, 18 W/840) from the top of the reactors. Lighting was operated on a 12 h light, 12 h dark period, and the light intensity was measured to be 180 µmol photons m^{-2} s^{-1} at the incident sides of the reactors by a light meter (840020C, Sper Scientific Ltd., USA). The experiment was operated in at a constant ambient temperature (23 ± 0.2 ˚C).

Table 4.1: Experimental conditions of two batch experiments, including the initial conditions of state variables in the model. X_{alg} and X_{bac} were quantified by TSS measurement. S_{CO2}, S_{HCO3} and S_{CO3} were calculated by TIC values and measured pH.

Initial conditions	**Batch 1 (pH 8.5)**		**Batch 2 (pH 7.5)**	
	Exp.1	**Exp.2**	**Exp.3**	**Exp.4**
$\boldsymbol{X_{alg}}$ **(mg L^{-1})**	90.0	90.0	86.6	86.6
$\boldsymbol{X_{bac}}$ **(mg L^{-1})**	1.00	9.10	1.00	8.60
Bacterial density (%)	1.1	9.2	1.1	9.0
$\boldsymbol{S_{CO2}}$ **(mg C L^{-1})**	0.13	0.13	1.24	1.24
$\boldsymbol{S_{HCO3}}$ **(mg C L^{-1})**	18.03	18.03	17.16	17.16
$\boldsymbol{S_{CO3}}$ **(mg C L^{-1})**	0.27	0.27	0.03	0.03

Samples were collected every 24 h from all reactors. Optical density at wavelength 680 nm (OD_{680}) was measured by a spectrophotometer (Varian Cary1 50, Varian, Inc., Australia) in a cuvette with a 1 cm light path. Dry algal biomass was determined gravimetrically by filtering 20 mL of culture through a GF/C filter (Whatman) which was rinsed with deionized water, dried in a 105 ˚C oven for 12 h, cooled in a silica gel desiccator and weighted to determine the algal dry weight. A linear relationship between OD_{680} and dry algal biomass was determined prior to the test. A separate study was conducted to verify that there was negligible interference from bacterial density to the OD

measurement of algal biomass within the time frame of this study. OD_{680} and algal biomass dry weight from five different culture densities was measured and correlated with a linear correlation (Equation 4.1), where m and n in the equation vary between different algal species. Preliminary tests established the linear correlation with $m = 342.91$ and$n = -10.19$, respectively at $R^2 = 0.98$. Bacteria did not affect the optical density measurement under 680 nm (tested in a separate study).

$$Dryweight\ of\ algae\ (mg\ DW\ L^{-1}) = m \times OD_{680} + n \qquad \text{Equation 4.1}$$

A simplified model describing algal growth based on Monod kinetics by inorganic carbon limitation was developed. Since algal growth in this study was limited by the availability of dissolved CO_2, other limiting effects were eliminated by ensuring sufficient nutrient availability, light, and enough trace elements and vitamins. The parameter estimations and model simulations were performed using the software package AQUASIM (Reichert, 1998). MATLAB (MathWorks, Inc., US) was applied to generate 3D-plots showing simulation results of algal biomass productivity R_{alg} (mg DW L^{-1} d^{-1}) with for a range of pH values (6-11) along a 10-day cultivation.

ii. Batch tests for verifying the application of the ABM on carbon dioxide and oxygen changes

Two photobioreactors were set up and a test protocol was established to simulate the growth of algae for verifying of the ABM on carbon dioxide and oxygen changes. 200 ml culture broth was started as working volume in the reactors. The magnetic stirs were kept at a constant speed to homogenize the culture. Light was generated by a portable fluorescent lamp from one side of the reactors. Lighting was operated on a 12 h light, 12 h dark period. The light intensity was measured to be 140 µmol photons m^{-2} s^{-1} at the incident sides of the reactors using the light meter. pH was natural and the initial pH was measured as 7.8±0.1. The ambient temperature was kept constant at 23˚C±0.2˚C, measured by a thermometer. Reactor 1 was supplied with 418 mg C L^{-1} $NaHCO_3$; while Reactor 2 was the control trial without adding external C source. Cultures were inoculated at 600 mg L^{-1} (on dry biomass basis).

Two membrane inlet mass spectrometer (MIMS) probes were set in Reactor 1 for detailed examinations of both O_2 and CO_2 fluxes associated with photosynthesis, one in the gas phase and the other in the liquid phase. Recorded mass to charge (m/z) ratio was 32 and 44 for O_2 and CO_2, respectively. The Hiden HPR-40 DSA dissolved species analyser bench top MIMS unit was used,

which contained a Hiden HAL 201 RC quadrupole mass spectrometer with dual faraday/electron multiplier detector and a mass range of 200 atomic mass units. MIMS unit inlets consist of a 4 way multistream selector for simultaneous sampling. Each MIMS probe had 0.5 m length, suited with silicon rubber membrane.

4.1.4 Free nitrous acid pre-treatment for Research Objectives 2 and 3

The FNA pre-treatment work plan comprised three batch tests (Table 4.2). Batch 1 and 2 were conducted for enhancing lipid/TAG recovery from algae (Research Objective 2); Batch 3 was conducted for enhancing methane production from algal digestion (Research Objective 3). After algae cultivation and lipids accumulation (Section 4.1.2), algal culture was concentrated to a paste by centrifugation in a Beckman Coulter, Allegra^{TM} X-12 at 3750 g for 3 min in 800 ml batches. In each batch, the collected algal pellet was re-suspended with de-ionized water and then the mixed liquor was evenly distributed between four beakers (reactor volume 500 ml) for FNA pre-treatment.

Table 4.2: Free nitrous acid pre-treatment experimental conditions applied in batch tests.

Batch	**Exp.**	**pH**	**NO_2^- (mg N L^{-1})**	**FNA (mg HNO_2-N L^{-1})**
Batch 1	pH6-FNA0	6	0	0
	pH6-FNA0.24	6	100	0.24
	pH6-FNA1.09	6	450	1.09
	pH6-FNA2.19	6	900	2.19
Batch 2	pH5-FNA0	5	0	0
	pH5-FNA2.25	5	100	2.25
	pH5-FNA13.49	5	600	13.49
	pH5-FNA26.98	5	1200	26.98
Batch 3	pH5.5-FNA0	5.5	0	0
	pH5.5-FNA0.77	5.5	100	0.77
	pH5.5-FNA2.31	5.5	300	2.31
	pH5.5-FNA14.61	5.5	1900	14.61

Predetermined amounts of sodium nitrite stock solution (30 g N L^{-1}) were added to the batch reactors in different volumes at the beginning of each experiment, which resulted in initial concentrations of

nitrite varying between 0 and 1900 mg NO_2-N L^{-1}. pH of the pre-treatment culture broth was maintained at a certain pH through manually adding 0.5 M HCl. All reactors were well mixed by magnetic stirring at constant speed of 350 rpm. The concentration of FNA was varied by controlling the nitrite concentration under pH 5, 5.5, or 6 as described in Table 4.2. Batch 1 and 2 was operated for investigating Research Objective 2, and Batch 3 was for Research Objective 3. One should note that, FNA could remain within the paste which has potential detrimental influence on the next step processing.

The FNA concentration was calculated using the following formula.

$$FNA\ (mg\ HNO_2\text{-}N\ L^{-1}) = S_{NO2^- - N}/(K_a \times 10^{pH}) \qquad \text{Equation 4.2}$$

where $S_{NO2^- - N}$ as dissolved NO_2^- concentration (mg NO_2-N L^{-1}) and $K_a = e^{-2,300/(273.15+T)}$ at an operation temperature T (ºC) (25ºC in this thesis) (Anthonisen et al., 1976).

In each batch test, mixed liquor samples were taken approximately every 12 hours using a syringe and immediately filtered through disposable Millipore filter units (0.22 μm pore size) for the off-line analysis. Cell membrane damage assay by fluorescent microscopy and scanning electron microscopy (SEM) observation was conducted before and after FNA pre-treatment to offer a further understanding on algal cell disruption and lipid distribution.

4.1.5 Lipid quantification, kinetic analysis, and fatty acids characterisation

iii. Lipid extraction and quantification

The algal liquor samples were taken from pre-treatment tests Batch 1 and 2 in duplicate. After the samples were taken, each sample was centrifuged at 3270 g (Beckman Coulter, Allegra™ X-12) for 3 min and the pellet was collected for lipid extraction analysis (in duplicate). Samples were taken from Batch 1 through the pre-treatment at 5 h, 24 h and 48 h and samples were taken from Batch 2 at the end of the pre-treatment (60 h).

A slightly modified version of Bligh and Dyer method (Bligh & Dyer, 1959) was applied for the total lipids extraction. Chloroform : methanol (1:1, v/v) was used as extraction solvent with the collected wet algal biomass. 3 g of wet algal paste was treated with 20 ml of solvent, followed by adding 10 ml de-ionised water. The bottom chloroform layer of the extracts was dried in vacuum until constant

weight. Moisture of algal pastes was determined by weighing certain amount of wet paste on filter paper (GF/C, Whatman) and dried in 105°C oven overnight, all in triplicates.

iv. Lipid extraction kinetic analysis

Additional to the Bligh and Dyer total lipid extraction, Soxhlet extraction apparatus with n-hexane as extraction solvent was applied to analyse the kinetic of lipid extraction. Lipid extraction kinetics of the treated algal biomass was studied by serial Soxhlet extraction apparatus (Wang & Weller, 2006) using n-hexane : ethanol (3:1, v/v) as extraction solvent. Lipid extraction yields were quantified for 2 h and 6 h extraction. The Soxhlet extractions were done in duplicate for each sample.

Parameters describing the lipid extraction process were determined by fitting the following first-order kinetic model to the experimental data using SigmaPlot® software (version 12; Systat Software, San Jose, CA, USA). This model was widely applied for the analysis of dissolution and leaching within liquid-solid systems (Fajardo et al., 2007; Halim et al., 2011):

$$Y(t) = Y_o(1 - e^{-kt}) \qquad \text{Equation 4.3}$$

where $Y(t)$ (wt% of dried algal biomass) is the lipid yield extracted by the organic solvent at time t, Y_o (wt% of dried algal biomass) is the amount of lipid originally present in the microalgal cells, k (h^{-1}) is the lipid mass transfer coefficient from the algal cells into the organic solvent (h^{-1}), t (h) is the extraction time.

All lipid extraction analysis was performed in duplicate. All mean values were analysed by one-way ANOVA and Tukey test (SigmaPlot® software) at p <0.05 to ditect significant differences.

v. Fatty acid characterisation

Since total lipid is a lumped measure of TAGs, free fatty acids, glycerolipids, as well as other "solvent-philic" compounds such as photosynthesis pigments. In making algal biodiesel, it is the TAG that matters since they are the feedstock which react with methanol in a reaction known as transesterification to produce FAMEs (Hu et al., 2008). Hence, the effect of FNA pre-treatment on TAG recovery from algal biomass was investigated. Six different FNA concentrations were considered. The relationship between the quantity and quality of the TAG recovered and FNA concentration was assessed. Additionally, the effect of FNA pre-treatment on different types of fatty acids was evaluated, with the view to identifying the optimum FNA concentration for lipid and TAG

recovery. GC-MS was applied to quantify and profile the fatty acids from the total lipids recovered.

Additionally, the quality of biodiesel that could be obtained from the recovered TAG was also evaluated considering the effect of FNA pre-treatment. The cetane number (CN) and the degree of unsaturation (DU) were determined by empirical equations (Equation 4.4 and Equation 4.5). Data from the literature were also used (Munack, 2006).

$$CN = 46.3 + \frac{5458}{SV} - 0.125 * IV \quad \text{Equation 4.4}$$

$$DU = MUFA + (2 \times PUFA) \quad \text{Equation 4.5}$$

where saponification value $(SV) = \sum \frac{(560 \times N)}{M}$, iodine value $(IV) = \sum \frac{(254 \times D \times N)}{M}$, D is the number of double bonds, M is the molecular mass and N is the percentage of each fatty acid component. MUFA and PUFA are their percentage of total fatty acids (% wt of total fatty acids).

4.1.6 Batch tests to determine anaerobic methane production

The following biochemical methane potential tests were undertaken in order to address the Research Objective 3 - Enhancing methane production from algal biomass using free nitrous acid pre-treatment.

vi. Anaerobic digestion material characteristics

Total chemical oxygen demand (TCOD), soluble chemical oxygen demand (SCOD), total solids (TS), volatile solids (VS) of the algae and inoculum were determined through triplicate measurements. The composition of the algae was also measured.

vii. Biochemical methane potential assay

The biochemical methane potential (BMP) assay provides a measure of the anaerobic biodegradability of a given substrate, and was used to determine the methane yields and process kinetics of the algal biomass with and without FNA pre-treatment (0-14.61 mg HNO_2-N L^{-1}, for 60 h), as described in Table 4.3. Algal biomass was batch digested in 240 ml sealed glass serum bottles (170 ml working volume). Batches were performed at an inoculum to substrate ratio of 1.5 (VS basis), where each BMP test contained 90 ml algal liquor and 80 ml inoculum. The digested sludge, used as inoculum, was degassed and kept under anaerobic conditions at 38±1 °C for 6 days prior to commencing the experiments. The anaerobic conditions were established by flushing the headspace

of each serum bottle with high purity nitrogen immediately followed by sealing with butyl rubber stopper secured by an aluminium crimp cap. The batches were incubated at 38±1 °C and agitated every 2-3 days. As shown in Table 4.3, the substrate of BMP-control was the algal liquor of the FNA-control trial; the substrate of BMP 1 was the algal pellet after FNA2.31 pre-treatment with the liquid removal by centrifuge; the substrate of BMP 2-4 was the pre-treated algae from FNA0.77, FNA2.31, and FNA14.61, respectively. Three sets of blanks (BLK-I, -II and -III) were also set up. BLK-I contained inoculum and Milli-Q water without algae. BLK-II and -III were identical to BLK-I except with the addition of nitrite stock solution, which resulted in an initial nitrite level of around 53 and 1006 mg N L^{-1} in BLK-II and -III, respectively. This was to evaluate the effect of nitrite on the performance of the inoculum. All tests were carried out in triplicates. The BMP tests lasted for 110 days, when biogas production dropped to insignificant levels. The biogas (CH_4, CO_2, H_2) production was monitored by subtracting biogas production from corresponding blanks. The methane yields were reported both on a COD basis (g CH_4-COD per g COD added) and a VS basis (L CH_4 per kg VS added) to make it easily comparable literatures.

Table 4.3: BMP tests conditions.

BMP tests	**Substrate source**[1]	**Initial NO_2 concentration (mg N L^{-1})**
BMP-control	Algae from FNA-control	0
BMP1	Algae from FNA2.31 with liquor removal	0
BMP2	Algae pre-treated liquor from FNA0.77	53
BMP3	Algae pre-treated liquor from FNA2.31	159
BMP4	Algae pre-treated liquor from FNA14.61	1006
BLK-I[2]	-	0
BLK-II[2]	-	53
BLK-III[2]	-	1006

[1] Algal came from FNA pre-treatment Batch 3

[2] No algae was added to the BLK series.

viii. Biochemical methane potential tests modelling

A two-substrate model was applied in this study (Equation 4.6). The key parameters associated with methane production from algae, the hydrolysis rates and the biodegradability, were modelled to evaluate and compare methane production rate and yield.

$$B(t) = B_{0,rapid}\left(1 - e^{-k_{rapid}t}\right) + B_{0,slow}(1 - e^{-k_{slow}t}) \quad \text{Equation 4.6}$$

where $B_{0,rapid}$ is the biochemical potential of the rapidly biodegradable substrates (g CH_4-COD per g COD added); $B_{0,slow}$ is the biochemical potential of the slowly biodegradable substrates (g CH_4-COD per g COD added); k_{rapid} is the hydrolysis rate of the rapidly biodegradable substrates (d^{-1}); and k_{slow} is the hydrolysis rate of the slowly biodegradable substrates (d^{-1}).

The parameters were determined by fitting the cumulative methane production data (average data from triplicate tests) from BMP tests to the kinetic model. The model was implemented in a modified version of Aquasim 2.1d (Batstone et al., 2009; Reichert, 1998).

4.1.7 Analytical methods and microscopy assay

The TS, VS, TCOD and SCOD were measured according to standard methods (American Public Health Association, 2005). TCOD and SCOD were measured using Spectroquant® photometric cell tests (114541 and 114555, Merk, Germany), a Thermoreactor TR 300 (Merck, Germany) and a UV-Visible Spectrophotometer (Varian Cary®50, Varian, Inc., Australia). NH_4^+-N, NO_3^--N, NO_2^--N, PO_4^{3-}-P, total Kjeldahl phosphorous (TKP), total Kjeldahl nitrogen (TKN) and total soluble Kjeldahl nitrogen (STKN) were measured with a Lachat QuikChem 8000 Flow Injection Analyser (Lachat Instrument, Milwau-kee, USA).

For Research Objective 1, in order to visually assesse the growth of algae and bacteria, fluorescent microscope observation on algal and bacterial cells was performed during cultivation. 1 ml of sample from every reactor was diluted 2 times before staining by adding 1 ml of Milli-Q water. 1 ml of diluted sample was stained with DAPI nuclear fluorescence microprobe (Invitrogen, Ltd., UK), according to a procedure modified from Porter and Feig (1980). Detailed procedure of microscopy assays is shown in Appendix A.

For Research Objective 2 and 3, in order to reveal the mechanism of FNA pre-treatment on the algal

cell disruption, cell membrane damage assay, SEM analysis and intracellular component release/solubilising was investigated.

Cell membrane damage assay was conducted by Sytox Green staining, which is a dye could only penetrate into damaged cell membrane and therefore allowing distinguish of intact and disrupted algal cells. Microscope observation and cell membrane damage assays were performed before and after pre-treatment. Untreated algae and algae samples from each reactor after 48 h pre-treatment were diluted 10 times before staining. SEM analysis was also performed as described by Naveena *et al.* (Naveena et al., 2005). Detailed procedure of microscopy assays is shown in Appendix C.

Intracellular component release/solubilising was investigated by tracing protein and polysaccharides concentration change during FNA pre-treatment. The protein concentration was measured by the bicinchoninic acid (BCA) method with Bovine serum albumin (BSA) as standard (Smith et al., 1985). The carbohydrate concentration was determined by the Anthrone method with glucose as standard (Raunkjær et al., 1994). The lipid content was measured by an InfraCal TOG/TPH Analyzer (Wilks Enterprise, Inc., USA) using S-318 as the extraction solvent.

5 RESEARCH OUTCOMES

This section summarises the research outcomes, which are covered in detail in journal papers that make up the Appendices section. The findings of Research Objective 1 have been submitted will be published in Biotechnology and Bioengineering. Some findings of Research Objective 2 have already been published in Bioresource Technology (Bai et al., 2014). The others have been accepted for publish in Applied Energy. The findings of Research Objective 3 has submitted for publication in Renewable Energy.

5.1.1 Research objective 1 (RO1) – Understand and model the contribution of bacteria to algal growth by carbon cycling in lab-scale algal culture system

ix. Contribution of bacteria to algal growth by carbon cycling

Carbon limitation is one of the major challenges for algal cultivation, and can greatly restrict algae productivity. In this thesis, a strategy based on bacteria carbon cycling to overcome carbon limitation is considered. Two batch tests, carried out at two different pH values, were conducted to examine the effect of the bacteria supplementation. Batch 1 was operated at pH 8.5 in triplicate for 7 days and Batch 2 was operated at pH 7.5 in duplicate and cultivation time was extended to 9 days. Algal growth was monitored through cultivation by measuring OD_{680} every 24 hours.

Normalized algal biomass accumulation through cultivation time is shown in Figure 5.1. For both batches (pH 7.5 and 8.5), a significant increase in algal growth was observed by adding heterotrophic bacteria. After 7 days of cultivation, algal productivity was 4.8 and 3.4 times higher in the systems supplemented with bacteria. The experimental results in this study suggest that carbon cycling by heterotrophic bacteria can achieve results similar to those seen for CO_2 supplementation.

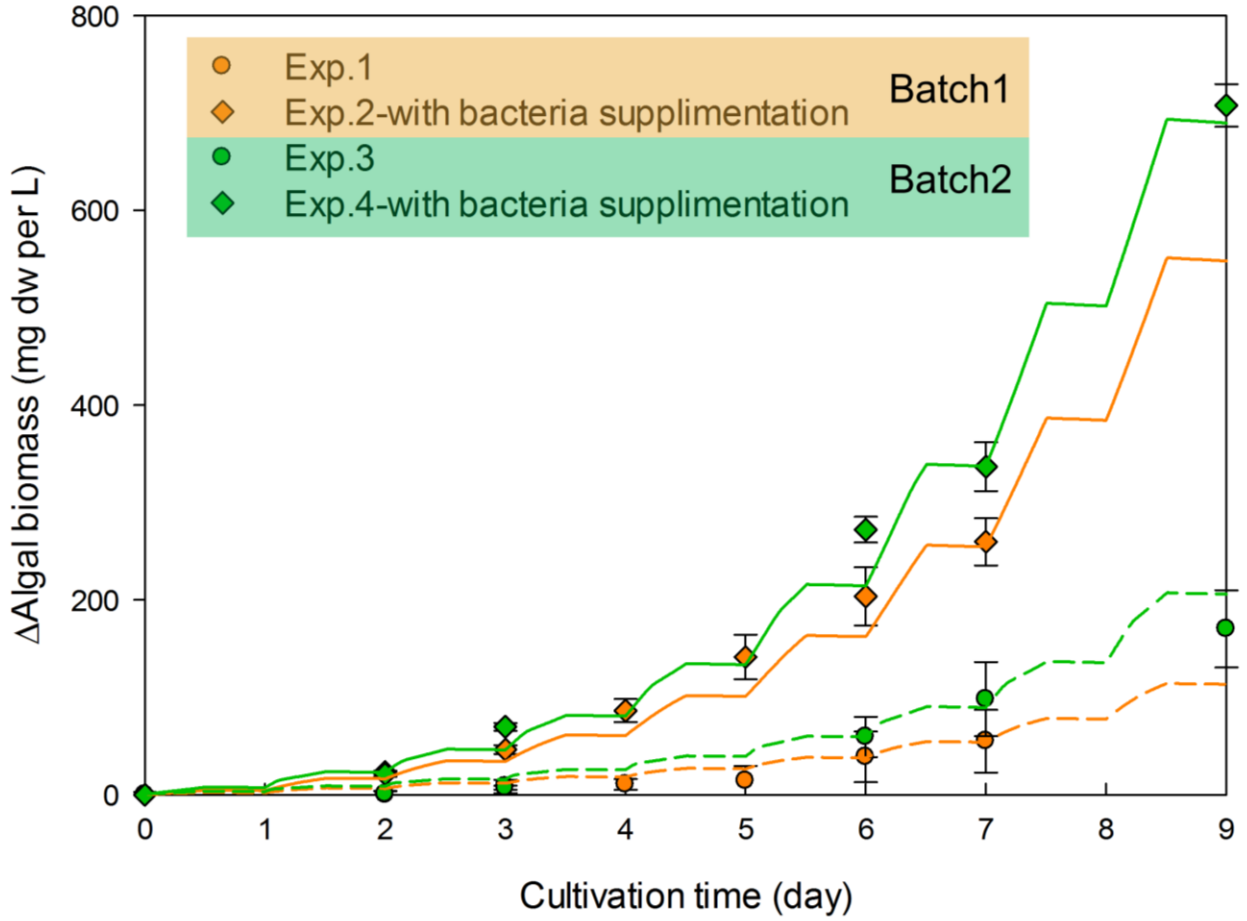


Figure 5.1 Experimental data (symbols) and model simulation results (lines) of algal productivity ΔX_{alg} Organge and green lines show the model simulation results of Batch 1 and Batch 2, respectively. Error bars show standard errors of Batch 1 (n=3) and Batch 2 (n=2), n is the number of samples.

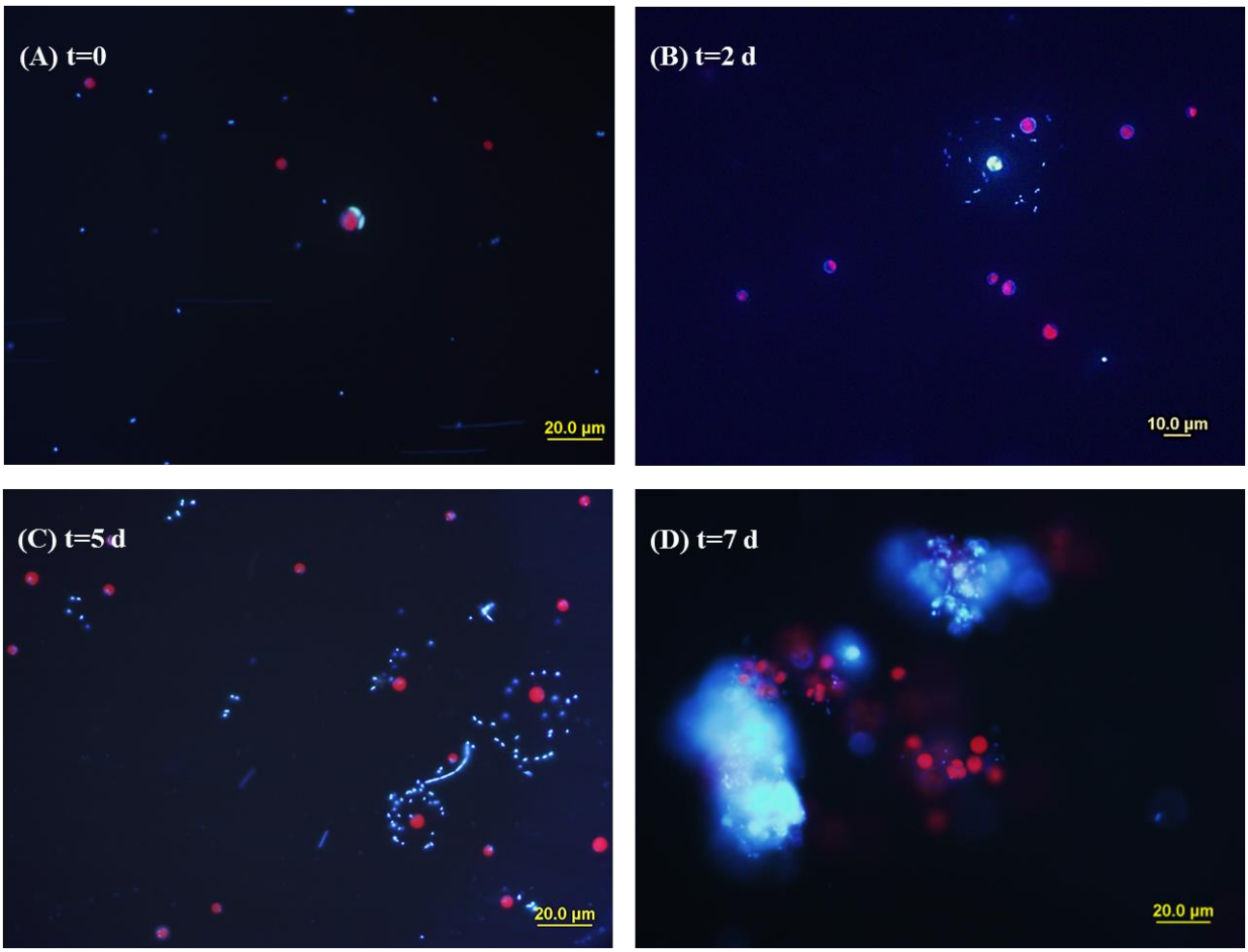


Figure 5.2 Flourescent microscope images of algal and bacteria cells from Exp.4 (Batch 2 with supplementary bacteria). (A) at the start of experiment, (B) at day 2, (C) at day 5 and (D) at day 7. Algae are flourescent red by chlorophyll autoflourescent. Bacteria are flourescent bright blue by UV excitation of DAPI stain.

It is apparent that algal productivity was higher at pH 7.5 than at pH 8.5. pH is an important component of the bicarbonate buffering system because it has a direct influence on the species of the inorganic carbon available to the algae. Increasing pH from 7.5 to 8.5 lowers the CO_2 pool from 6.7 % to 0.7 % of the total dissolved inorganic carbon (DIC).

It is acknowledged that the majority of algae (all cyanobacteria and most of green eukaryotic algae) have developed carbon concentrating mechanisms (CCMs) as an evolutionary response to low CO_2 levels. For example, they can convert HCO_3^- to CO_2 by carbonic anhydrase activity, resulting in an internal Ci (internal inorganic carbon concentration) of 5 to even 75-fold higher than that of the

surrounding medium (Wang et al., 2011). But it has been shown that the *Chlorella* sp. applied in this study has a negligible affinity for HCO_3^- as a growth substrate (Keymer et al., 2013a). A possible explanation for this is the lack of carbonic anhydrase (CA) in this algae, preventing it from converting HCO_3^- to CO_2 (Rotatore & Colman, 1991a; Rotatore & Colman, 1991b). Thus the activity of the algae used in this study is particularly sensitive to CO_2, which is why the activity was markedly higher at the lower pH.

Fluorescent microscope images (Figure 5.2) show the algal and bacterial cells from bacteria supplemented test through cultivation. After 7 days, the amount of algal cells increased, which was in line with the OD_{680} measurement results. Comparing Figure 5.2A and Figure 5.2D shows that a significant amount of bacterial growth occurred. Algal and bacterial cells were tending to form flocs in the culture through the co-cultivation. The natural bio-flocculation process of algal and bacterial biomass has been reported by several studies and applied to some wastewater treatment processes (Vadivelu et al., 2006; Van Den Hende et al., 2011a). A benefit of forming flocs is that bacterial cells may increase the local concentration of dissolved CO_2 for algal cells.

x. Model based analysis of the contribution of bacteria on carbon cycling

Figure 5.3 illustrates the conceptual model developed for this research objective. The model consists of 5 state variables (S_{CO2}, S_{HCO3}, S_{CO3}, X_{alg}, X_{bac}), 2 biological algal processes (growth and death of algae), 1 bacterial process (net growth of bacteria), as well as 3 chemical-physical processes. A simplified model, with process and rate expressions, is presented in the Petersen matrix in Table 5.1. Detailed process description can be found in Appendix A.

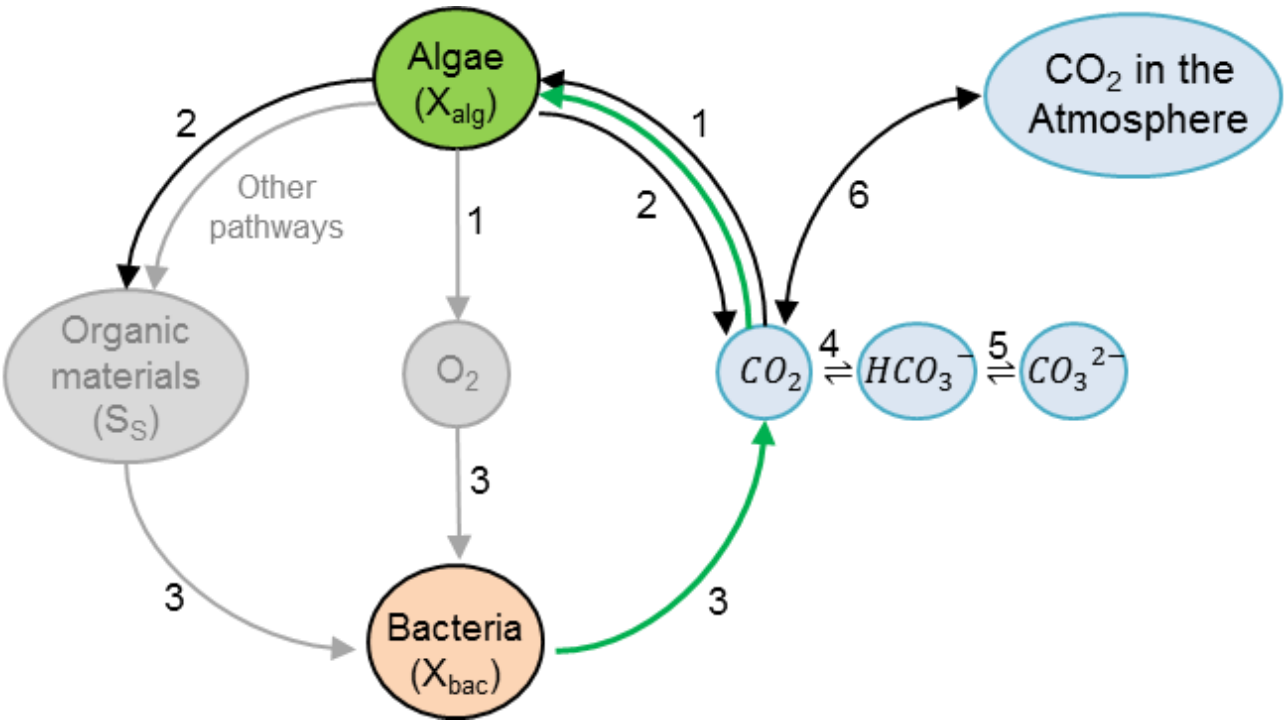


Figure 5.3 Conceptual model of carbon recycle by bacteria in open algal culture systems. Process 1, the growth of algae, is described as photosynthesis by algae and is limited by the availability of CO_2 (S_{CO2}) and light intensity. Death of algae (Process 2) is the process of conversion of algal biomass (X_{alg}) to slowly degradable and other organic matter by decay and lysis. Net bacterial growth is presented as Process 3. Processes 4-6 represent gas-liquid mass transfer of CO_2 and chemical equilibria of the bicarbonate system.

Table 5.1: Petersen's matrix for the applied model in this study.

Component *i* →		**1**	**2**	**3**	**4**	**5**	**Rates**
Process *j* ↓		**S_{CO2}**	**S_{HCO3}**	**S_{CO3}**	**X_{alg}**	**X_{bac}**	
1	Growth of algae with CO_2	$-a_{C,alg}$ (1)			1		$\mu_{max,alg}\frac{S_{CO2}}{S_{CO2}+K_{CO2,alg}}\frac{I_{ave}}{I_{opt}}e^{\left(1-\frac{I_{ave}}{I_{opt}}\right)}X_{alg}$
2	Death of algae	$a_{C,alg}$-0.21 (2)			-1		$b_{alg}X_{alg}$
3	Net growth of bacteria (3)	0.43				1	$\mu_{bac}X_{bac}$
4	Equilibrium $CO_2 \rightarrow HCO_3^-$	-1	1				$k1 \times \left(S_{CO2} - S_H \times \frac{S_{HCO3}}{K_{eq1}}\right)$ (4)
5	Equilibrium $HCO_3^- \rightarrow CO_3^{2-}$		-1	1			$k2 \times \left(S_{HCO3} - S_H \times \frac{S_{CO3}}{K_{eq2}}\right)$ (5)
6	Mass transfer of CO_2	1					$K_{LaCO2}(S^*_{CO2} - S_{CO2})$ (6)

(1) Mass fraction of algae was measured in this study by elemental analysis.

(2) Stoichiometric parameter of death of algae was determined by the carbon mass fraction of algal biomass and the conversion of algal biomass to inert particulate organic material or biodegradable particulate organic material (Reichert et al., 2001b).

(3) This process was only activated in the bacteria supplemented trials. Net growth rate of bacteria μ_{bac} lumped the growth of bacteria and the death of bacteria.

(4) $K_{eq1} = S_{HCO3} \cdot \frac{S_H}{S_{CO2}}$, $S_H = 10^{-pH}$.

(5) $K_{eq2} = S_{CO3} \cdot \frac{S_H}{S_{HCO3}}$.

(6) $\mathrm{KLa_{CO2}} = \mathrm{KLa_{O2}}\sqrt{\frac{\mathrm{D_{CO2}}}{\mathrm{D_{O2}}}}$. $\mathrm{KLa_{O2}}$, the mass transfer coefficient of O_2, was obtained by the dynamic method of measuring DO with an optical DO probe (AquaPlus, Aquaread Ltd., UK) (Torres-Martínez et al., 2009). $\mathrm{D_{CO2}}$ and $\mathrm{D_{O2}}$ as 1.97×10^{-9} and 2.1×10^{-9} d^{-1} are the diffusion coefficient of CO_2 and O_2 in water respectively at 25 ºC (Wolf et al., 2007). S^*_{CO2} (mg C L^{-1}) is the saturated CO_2 concentration in the solution. It is calculated from the atmospheric concentration of CO_2 and the Henry constant (Sander, 1999).

Table 5.2: Estimated parameters from experimental data fitting to the model.

Parameter	Definition	Value	Units
$\mu_{max,alg}$	Maximum specific growth rate of algae	0.89 (0.11-1.9)	d^{-1}
μ_{bac}	Specific growth rate of bacteria	0.68 (0.65-0.69)	d^{-1}

Table 5.2 defines two key parameters, $\mu_{max,alg}$ and μ_{bac} estimated in this study using the experimental data using a least squares parameter estimation.

Figure 5.4 was plotted to give an overview of what one may expect for a range of pH values and the effect of the presence of bacteria in the culture system. When bacteria is not present in the culture (Figure 5.4A), a plateau appears at 0.8 ($\log_{10}$ mg DW L^{-1} d^{-1}), indicating a limitation of the algal biomass productivity by S_{CO2}. However, when bacteria are supplemented in the system (Figure 5.4B), this limitation is no longer present due to bacteria recycling carbon and making it available for algal growth.

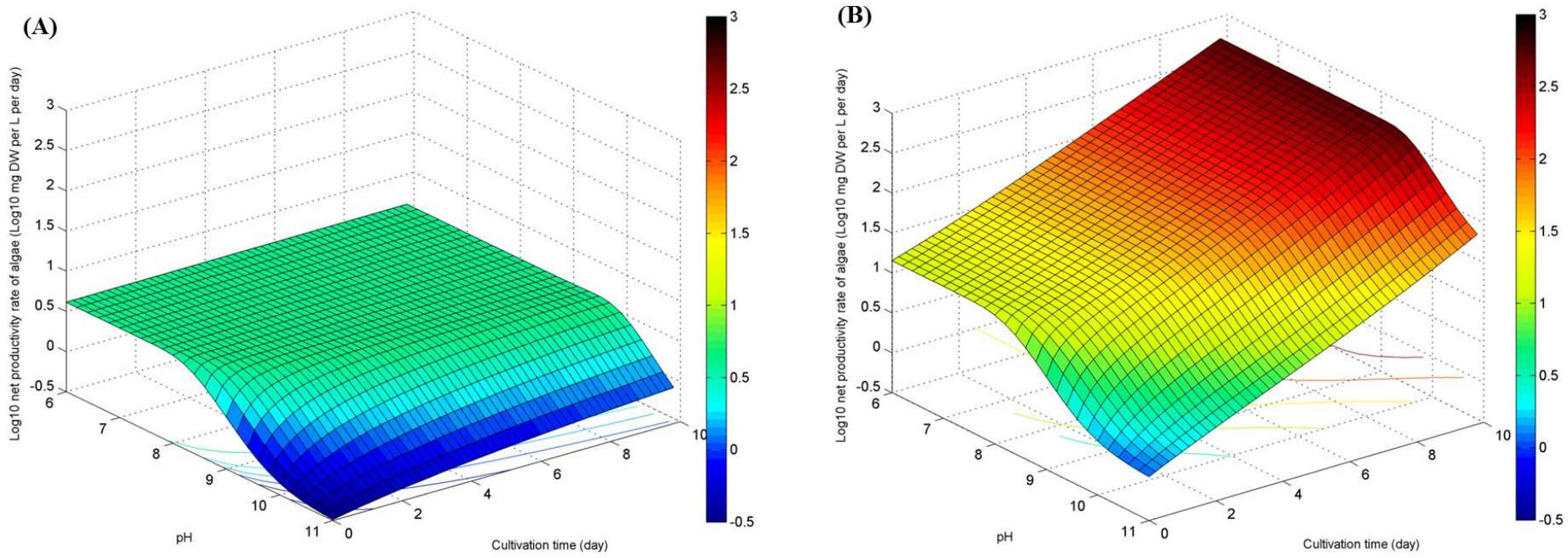


Figure 5.4 Example surface plots showing the model fits over a range of pH values using initial conditions measured and parameters estimated from this study: (A) without bacteria supplementation; (B) with bacteria supplementation.

xi. Expanded Algae-Bacteria Model

The simplified model developed in Section 5.1.1x is expanded to consider the major processes related to C, O and N. Figure 5.5 provides an overview of the Algae-bacteria model (ABM) which considers the comprehensive carbon and nutrient fluxes in open algal systems, considering the activity of heterotrophic bacteria. The entire brochure of the model description and application method is present in Appendix B.

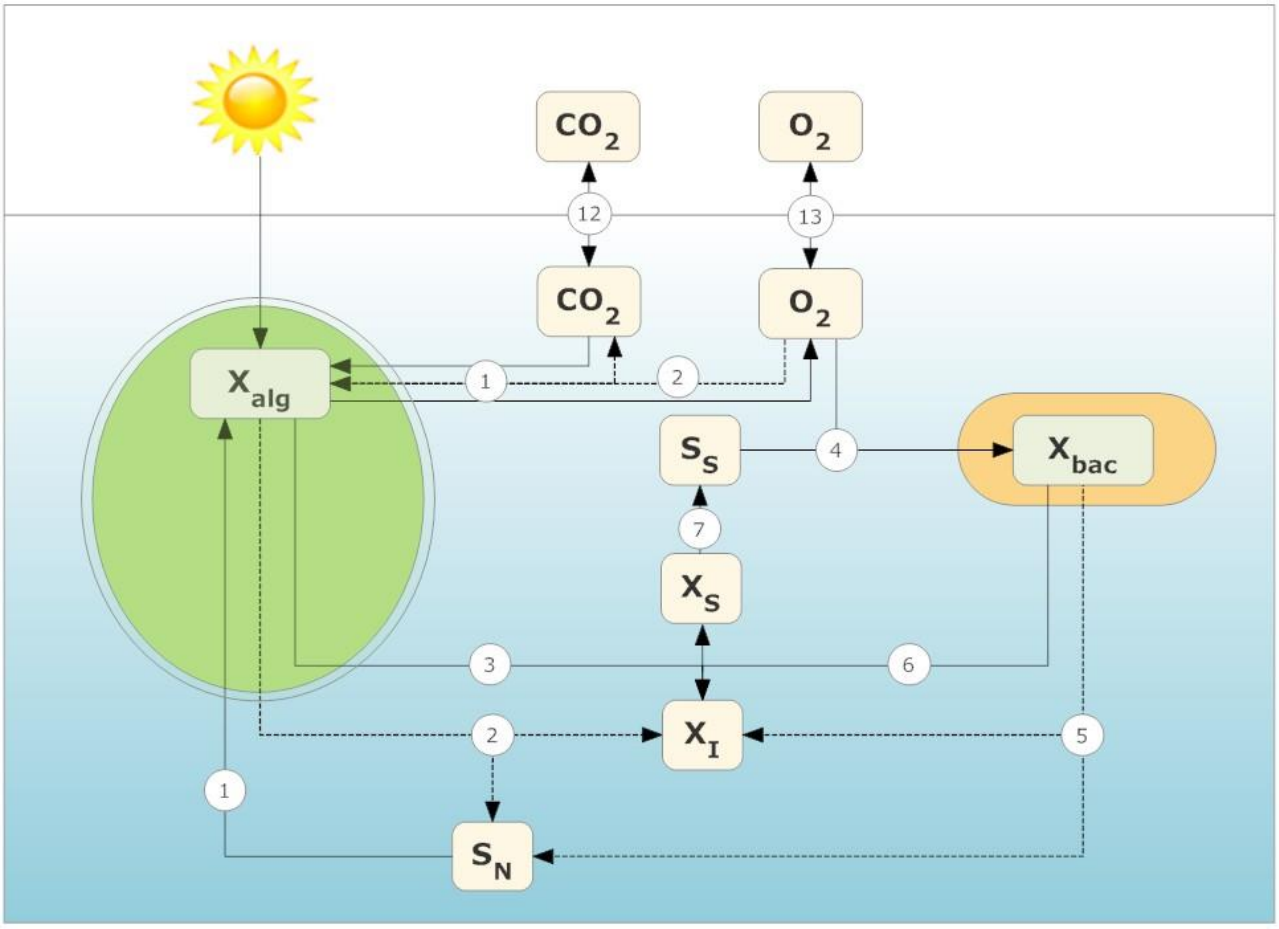


Figure 5.5 Schematic diagram of carbon flows in Algae-Bacteria Model. Numbers in the model represent biological and chemical processes.

ABM describes the relationship among four solid phase species: algal biomass and bacterial biomass, and slowly biodegradable organic matter, non-biodegradable organic matter; ten soluble species: readily biodegradable organic matter, nitrate, ammonium, ammonia, dissolved carbon dioxide, carbonate, bicarbonate, dissolved oxygen, hydrogen ion, and hydroxide ion. The model involves seven microbial processes and six chemical processes. The model is derived on a carbon basis; Figure 5.6 gives a clear description on the carbon components in the model. Total carbon (TC) is divided into soluble (*S*) and particulate (*X*) components. The total carbon is further subdivided into total

inorganic carbon (TIC), X_{bac}, X_{alg}, X_I, X_S and S_S. The biodegradable matter is divided into S_S and X_S. X_{bac} and X_{alg} are generated by growth on S_S and TIC respectively.

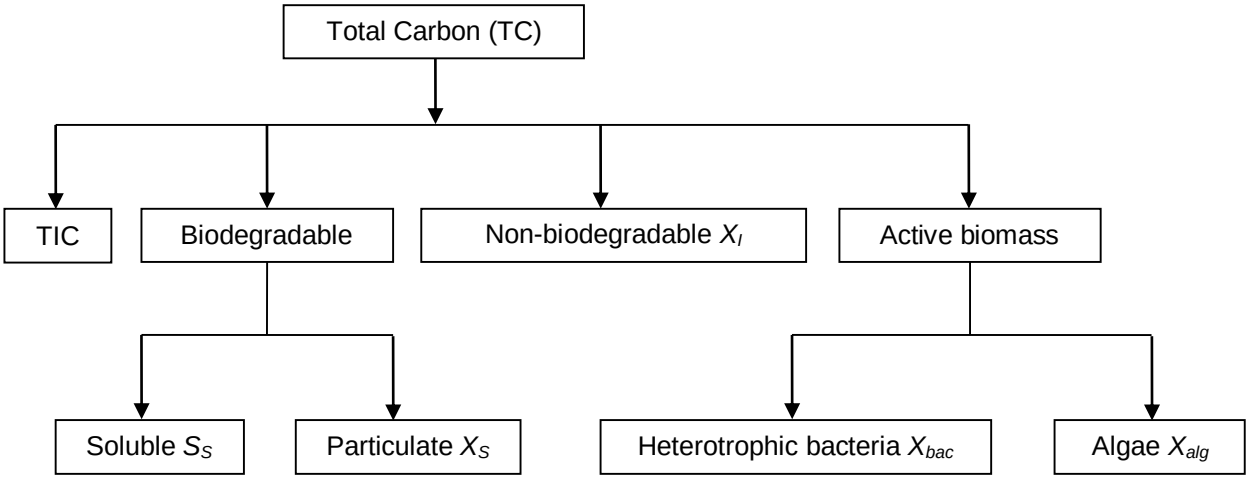


Figure 5.6 Carbon components in the algae-bacteria model.

The structure of the established ABM being presented in a matrix format is shown in Table 5.3, showing the basic stoichiometric parameters (stoichiometric parameter definitions are shown in Appendix B), as well as the kinetics rate expression of each process. In the model matrix, model components (state variables) are listed in the first row and relevant stoichiometric coefficients are incorporated in appropriate cells of the matrix. The leftmost column gives the description of processes and the rightmost column describes the process rates (Gujer et al., 1995). Process 1, 3-6, 9, 10 and 12 of the developed ABM was applied into evaluating the carbon cycling by heterotrophic bacteria, which is part of key outcomes from Research Objective 1.

The Petersen's matrix format enables the mass balance for each state variable to be easily written. As shown in Equation 3. 1, the mass balance of component i is obtained by summing the products of the stoichiometric coefficients ϑ_{ij} and the process rate expressions r_j. For example, the accumulation algal biomass (X_{alg}) is obtained by considering the growth of algae and the death of algae (Equation 3. 2).

$$\frac{di(t)}{dt} = \sum_j \vartheta_{ij} \cdot r_j \qquad \text{Equation 3. 1}$$

$$\frac{dX_{alg}(t)}{dt} = \mu_{max\_alg} \frac{S_{CO2}}{S_{CO2}+K_{CO2,alg}} \frac{S_{NO3}}{S_{NO3}+K_{NO3,alg}} \frac{I_{ave}}{I_{opt}} e^{\left(1-\frac{I_{ave}}{I_{opt}}\right)} X_{alg} X_{alg}(t) - k_{resp,alg} \frac{S_{O2}}{K_{O2,alg}+S_{O2}} X_{alg}(t) - b_{alg} X_{alg}(t) \qquad \text{Equation 3. 2}$$

Table 5.3: Stoichiometric matrix for the established Algae-Bacteria Model.

	Component $i \rightarrow$	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**Rates**
	Process $j \downarrow$	S_S	S_{CO2}	S_{HCO3}	S_{CO3}	S_{O2}	S_{NO3}	S_{NH4}	S_H	S_{OH}	X_{alg}	X_{bac}	X_S	X_I	
1	Growth of algae with CO_2		-0.52			1.62	-0.026		-0.0018		1.00				$\mu_{max,alg}\frac{S_{CO2}}{S_{CO2}+K_{CO2,alg}}\frac{S_{NO3}}{S_{NO3}+K_{NO3,alg}}\frac{I_{ave}}{I_{opt}}e^{(1-\frac{I_{ave}}{I_{opt}})}X_{alg}$
2	Respiration of algae		0.40			-1.49		0.02	0.01		-1.00			0.20	$k_{resp,alg}\frac{S_{O2}}{K_{O2,alg}+S_{O2}}X_{alg}$
3	Death of algae		0.16			-0.40		-0.01	0.00		-1.00		0.50	0.12	$b_{alg}X_{alg}$
4	Growth of bacteria	-1.67	0.43			-1.39		-0.0033	0.00024			1.00			$\mu_{max,bac}\frac{S_S}{S_S+K_S}\frac{S_{NH4,bac}}{S_{NH4,bac}+K_{NH4,bac}}\frac{S_{O2}}{S_{O2}+K_{O2,bac}}X_{bac}$
5	Respiration of bacteria		0.40			-1.59		0.11	0.01			-1.00		0.20	$k_{resp,bac}\frac{S_{O2}}{S_{O2}+K_{O2,bac}}X_{bac}$
6	Death of bacteria		0.16			-0.44		0.08	-0.01			-1.00	0.50	0.12	$b_{bac}X_{bac}$
7	Hydrolysis	1.00											-1.00		$k_{hyd}X_S$
8	Equilibrium $H_2O \rightarrow H^+ + OH^-$								1.00	1.00					$1 - S_H S_{OH}/K_{eq,w}$
9	Equilibrium $CO_2 \rightarrow HCO_3^-$		-1.00	1.00											$k1 \times \left(S_{CO2} - S_H \times \frac{S_{HCO3}}{K_{eq1}}\right)$
10	Equilibrium $HCO_3^- \rightarrow CO_3^{2-}$			-1.00	1.00										$k2 \times \left(S_{HCO3} - S_H \times \frac{S_{CO3}}{K_{eq2}}\right)$
11	Equilibrium $NH_4^+ \rightarrow NH_3$														$S_{NH4} - S_{NH3}S_H/K_{eq3}$

12	Gas-liquid mass transfer of O_2		1.00	$K_{LaCO2}(S^*_{CO2} - S_{CO2})$
13	Gas-liquid mass transfer of CO_2	1.00		$K_{LaO2}(S^*_{O2} - S_{O2})$

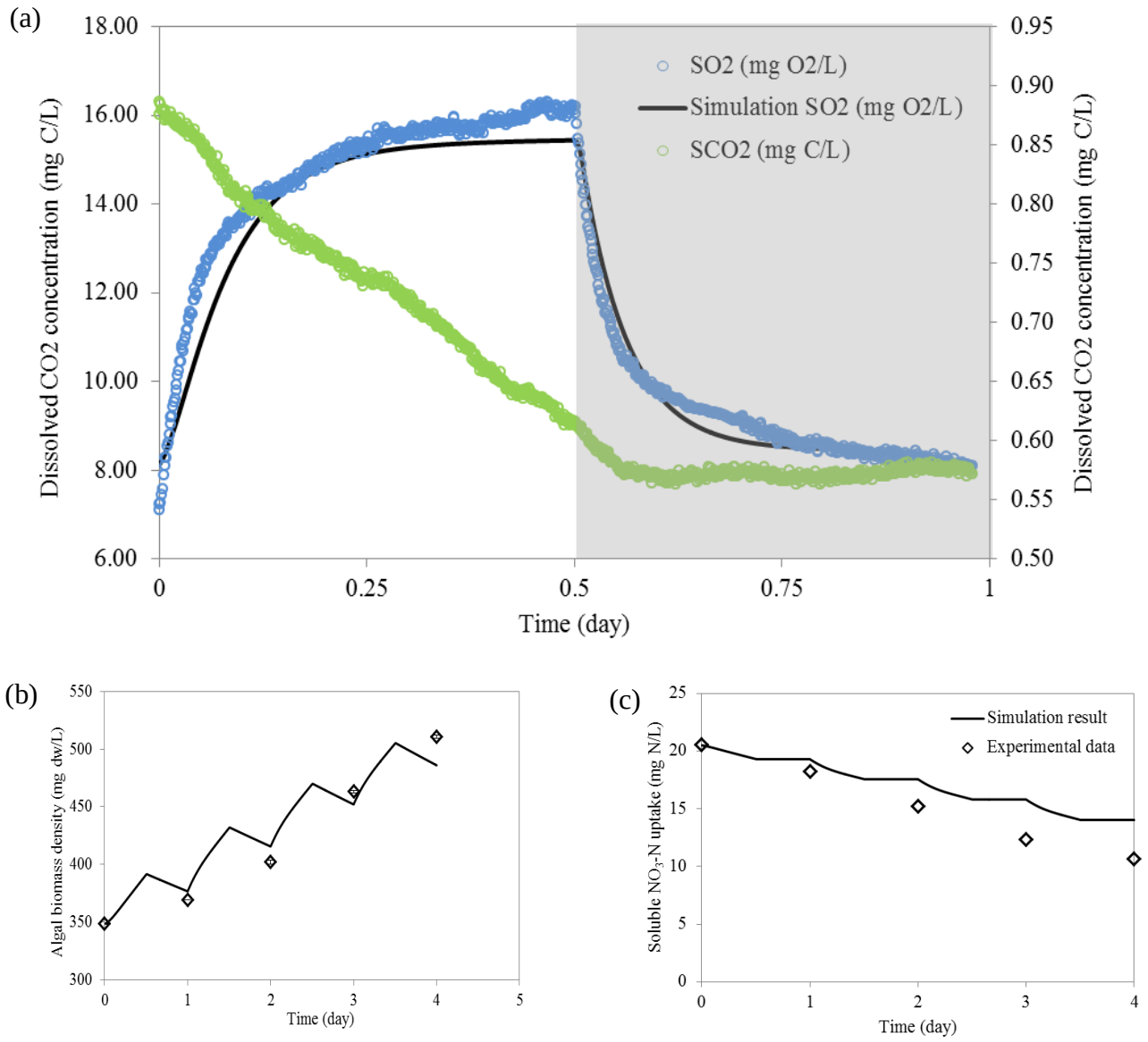


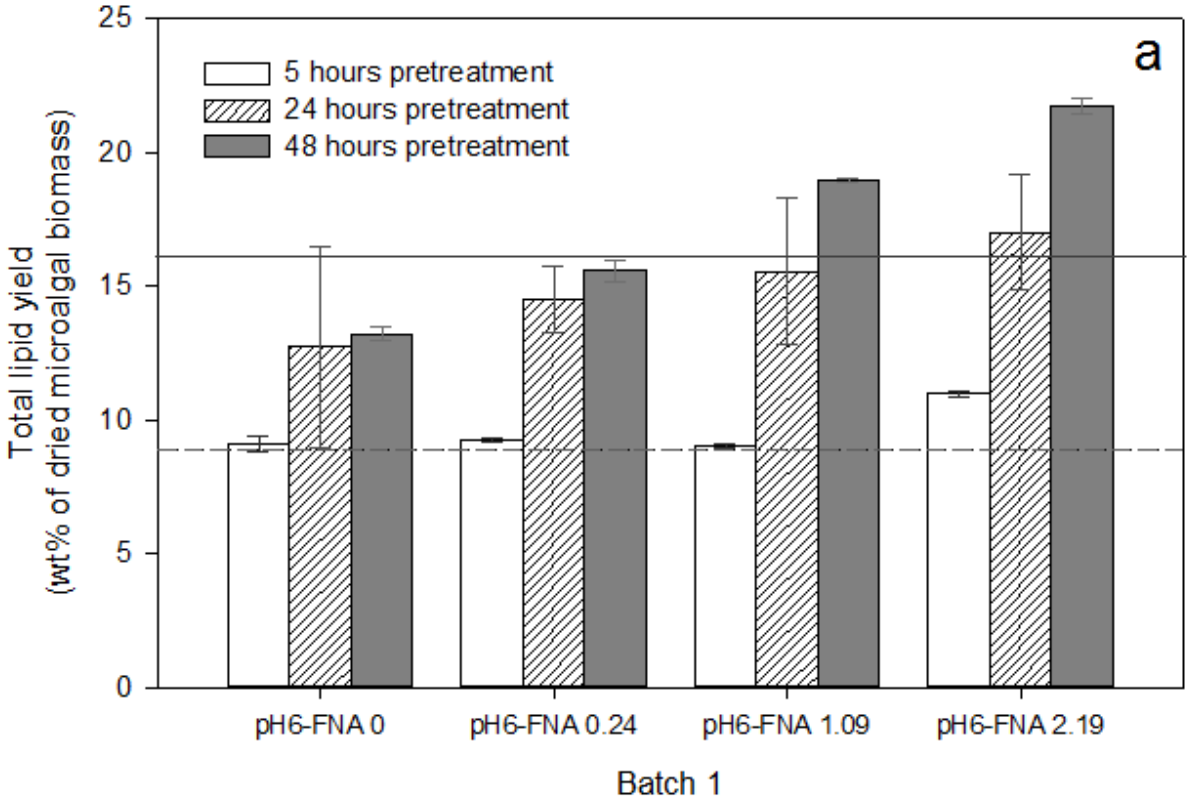
Figure 5.7 Experimental and model simulation results.

Figure 5.7 provides a general view on the model application. The model (solid line) was fitted to one data set of dissolved oxygen concentration and dissolved carbon dioxide change during one diurnal cycle, which resulted in the estimated parameter values. Model curves obtained with the same parameter values are shown for other two data sets: Figure 5.7b represents the growth of algae in 4 days and Figure 5.7c shows the soluble NO_3-N consumption by algae. The model simulation results present a well-predicted algae growth. Yet predicted the nitrogen assumption by algae (Figure 5.7c) is slower than the experimental results. This is likely because algae has the ability to rapidly accumulate nitrogen as intracellular nitrogen pool. The model is limited to relatively short term growth experiments; to model long term batch cultivation other factors, like the production and accumulation of toxic by-products, would have to be considered.

5.1.2 Research objective 2 (RO2) – Enhance lipid, and specifically triacylglyceride (TAG), extraction from algae by using free nitrous acid pre-treatment

xii. FNA pre-treatment enhanced lipid recovery yield

Lipid extraction is limited by the rigid cell envelope (cell membrane and cell wall) poses by algae. In this thesis, a strategy to disrupt cells based on FNA pre-treatment is considered. Results of the lipid extraction by Bligh & Dyer method after FNA pre-treatment are shown in Figure 5.8. In line with the hypothesis (Section 3.1.2), lipid extraction yield was boosted dramatically for all experiments (after 48 hours pre-treatment) compared to untreated algal biomass. Lipid yields after 48 h pre-treatment kept increasing; meanwhile the degree of increase was heightened by increasing FNA concentration. For pH6-FNA1.09 and pH6-FNA2.19 trials, yielded lipid content reached 19.0±0.1 % and 21.9±0.2 % (wt % of dried algal biomass) respectively, a 2.1-fold and 2.4-fold increase over the untreated algae (pH6-FNA0), which confirms the great potential of using FNA as novel pre-treatment technique for lipid extraction from algal biomass. An extended pre-treatment (60 h) was trialled for Batch 2 (Table 4.2). Figure 5.8b shows that different concentrations of FNA, higher than 2.25 mg HNO_2-N L^{-1} up to 26.98 mg HNO_2-N L^{-1}, all had a similar effect on lipid extraction, which boosted the lipid extraction yield 2.5-fold in comparison with that from untreated algal biomass (dashed line). The results from Batch 2 suggest that continuing to increase FNA concentration and pre-treatment time does not significantly improve lipid extraction; 24.4±0.3 % (wt % of dried algal biomass) is approaching the lipid content of algal biomass of Batch 2.



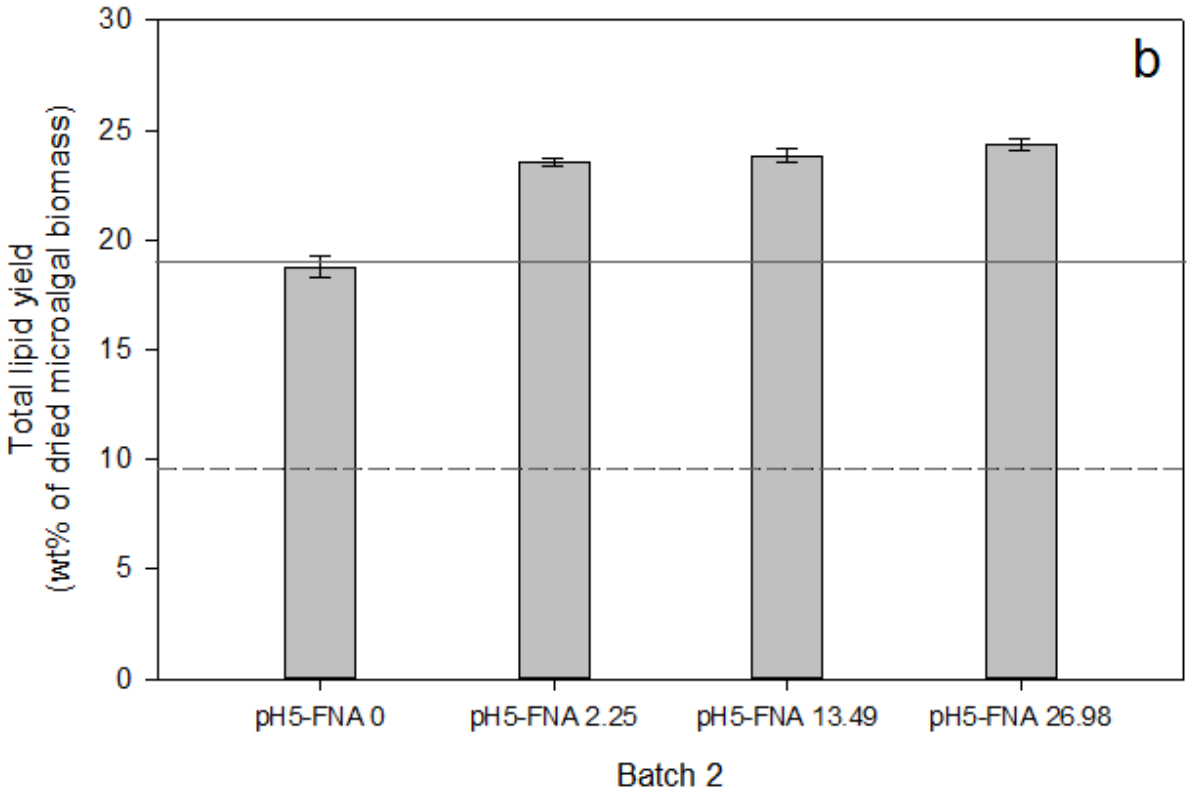


Figure 5.8 Results of lipid yields by Bligh and Dyer extraction in different experiments. Error bars indicate standard error in duplicates tests: (a) results from batch 1; (b) results from batch 2. Dashed lines show lipid yields from untreated algal biomass; solid lines show lipid yields from microwaved algal biomass. Error bars are standard errors, n=2.

xiii. FNA pre-treatment enhanced lipid extraction kinetics

Although the Bligh and Dyer method has been widely applied for the majority of algal lipid analysis in the lab, the application of n-hexane, with lower toxicity and more potential for industrial scale-up should be considered. Hence, the dynamic hexane extraction by Soxhlet apparatus was carried out to analyse the kinetic of lipid extraction.

The n-hexane lipid extraction results, with best-fit of the first-order kinetic models (Section 4.1.5iv) are shown in Figure 5.9. Table 5.4 summarizes extraction parameter k (h^{-1}), which is the lipid mass transfer coefficient (from the algal cells into the organic solvent). As the FNA concentration increased, the mass transfer coefficient k increased from 0.39 h^{-1} to 0.96 h^{-1}. This significant increase in mass transfer coefficient supports that the intracellular lipids became more readily available for solvent extraction with increasing levels of FNA pre-treatment. Moreover, the average solvent recovery was 72.6±0.0 % and 64.4±0.0 % after 2 h and 6 h extraction respectively. And an increasing trend of solvent recovery can also be revealed by increasing FNA concentration pre-treatment, which indicates more efficient solvent utilization with higher level of FNA pre-treatment.

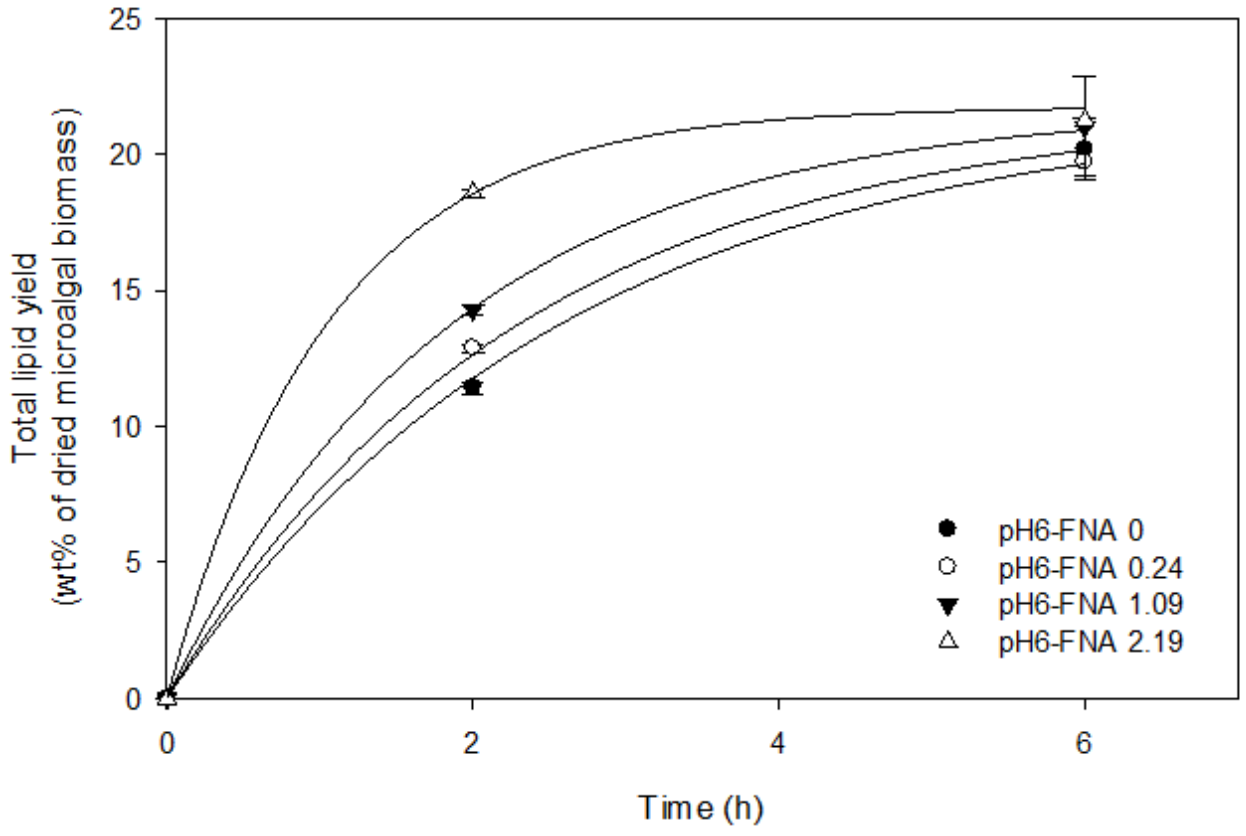


Figure 5.9 Results of lipid yields by Soxhlet extraction. Lines show the first order kinetic fitting curves. Error bars are standard errors, n=2.

Table 5.4: Lipid Soxhlet extraction kinetics parameters and solvent recovery (mean ± standard error).

Exp.	k (h^{-1})	R^2	r_2 (% solvent recovered after 2 h extraction)	r_6 (% solvent recovered after 6 h extraction)
pH6-FNA 0	0.39±0.02	0.9980	69.2±0.0	61.5±0.0
pH6-FNA 0.24	0.43±0.02	0.9988	72.1±0.0	63.5±0.0
pH6-FNA 1.09	0.54±0.01	0.9999	73.1±0.0	65.4±0.0
pH6-FNA 2.19	0.96±0.05	0.9993	76.0±0.0	67.3±0.1

xiv. FNA pre-treatment enhanced triacylglyceride recovery

Section 5.1.2 xii and xiii reveals that total lipid (TL) extraction can be enhanced dramatically by FNA pre-treatment. However, data on the amount triacylglyceride (TAG) among total lipid recovered and the characteristics of the fatty acid profile are vital for the production of biodiesel. Therefore, in this thesis, the effect of FNA pre-treatment on TAG recovery from algal biomass is addressed.

As shown in Figure 5.10, for a range of low FNA concentration pre-treatments (0.24-2.25 mg HNO_2-N L^{-1}), an increase in FNA concentration boosted TAG recovery along with total lipid recovery. A maximum 3.3-fold enhancement in TAG recovery from FNA-2.19 over the untreated algae was observed. However, although higher FNA concentration (13.49 and 26.98 mg HNO_2-N L^{-1}) remained effective for recovery of total lipids, it was detrimental for TAG recovery. With 13.49 and 26.98 mg HNO_2-N L^{-1} FNA pre-treatment, TAG recovery was significantly lower than for low range FNA pre-treatment and only slightly higher than it was for untreated algae. Optimum FNA concentration was around 2 mg HNO_2-N L^{-1} for the TAG recovery.

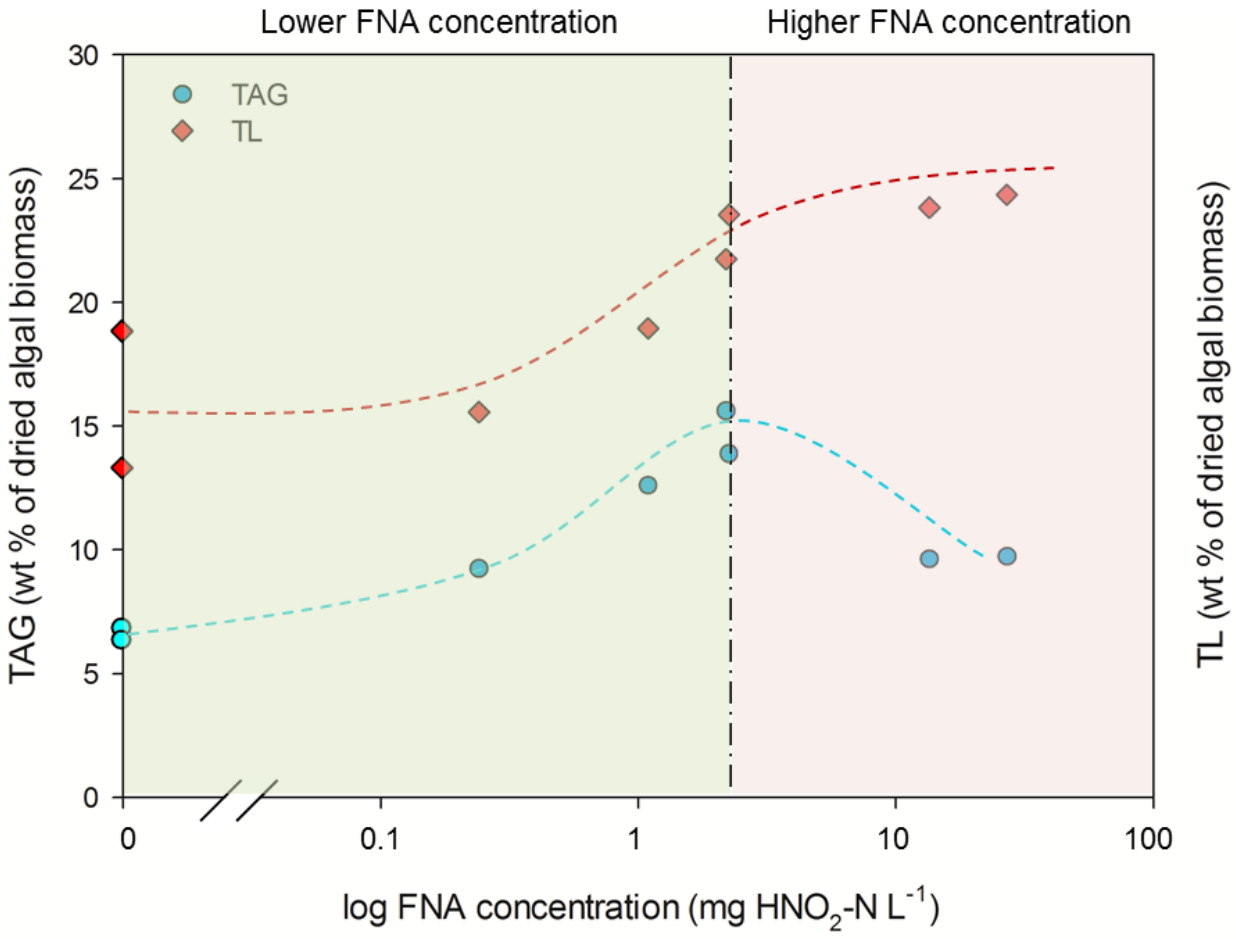


Figure 5.10 Total lipid (TL) and TAG recovery after different levels of FNA pre-treatment.

The properties of biodiesel that are related to the fatty acid profile include cetane number CN and oxidative stability (measured as the degree of unsaturation (DU)). CN is a dimensionless descriptor of the ignition quality of diesel. The CN value of the reference fuel cetane is 100. Standard ASTM D6751 (Materials, 2009) for biodiesel fuel requires a minimum CN of 47. In Table 5.5, the CN value of the TAG recovered improved to >47 after FNA pre-treatment, comparing to 44 in untreated algae. The degree of unsaturation (DU) is another measure of the physical and fuel properties of the biodiesel. The values obtained varied from 112.5 to 147.0 after different levels of FNA pre-treatment, which were lower than, and therefore an improvement over, the control (DU=153.9).

After FNA pre-treatment, the major detected fatty acids did not change, but the relative amounts of each fatty acid did vary. As seen from Figure 5.11 (A-C), for a range of low FNA pre-treatments (0.24-2.25 mg HNO_2-N L^{-1}), the amount of SFA, MUFA and PUFA being recovered was boosted with increasing FNA concentration. However, higher range FNA pre-treatments (13.49 and 26.98 mg HNO_2-N L^{-1}) showed negative effect on fatty acids recovery (Figure 5.11). As shown in Table 2, with higher range FNA pre-treatments (13.49 and 26.98 mg HNO_2-N L^{-1}), although there was an enhancement in C16, C18:1, C18:2 and C22:1 recovery over untreated algae, the recovery of the other fatty acids was largely reduced, especially the recovery of polyunsaturated fatty acids (eg, C16:3, C16:4 and C20:4). It has been reported that active groups of FNA ionized system such as NO• (nitric oxide) and NO_2 can induce lipid peroxidation (Hogg & Kalyanaraman, 1999). Therefore, it is very likely that the reason for the negative effect of high FNA concentrations (13.49 up to 26.98 mg HNO_2-N L^{-1}) on PUFA recovery is because that unsaturated fatty acids were damaged or oxidised by NO• and NO_2.

In addition to generating biodiesel, the essential fatty acids (EFAs) as valuable food supplement are of particular interest (Arts et al., 2001). Comparing to untreated algae, C20:4 (arachidonic, ω6) recovery kept increasing as FNA concentration increased up to 2.25 mg HNO_2-N L^{-1}. The highest C20:4 was detected as 1.65 (wt% of dry algal biomass), which was a 3.3 times increase than the untreated algae.

.

Table 5.5: Total lipids (TL), total fatty acid (FA), total saturated fatty acid (SFA), total unsaturated fatty acid (USFA), total mono unsaturated fatty acid (MUFA), and total poly unsaturated fatty acid (PUFA), and biodiesel quality parameters. All the fatty acid data is shown as wt % of dry algal biomass (ash containing dry algal biomass), and crude lipids data are shown as mean (± error), n=2.

	Untreated algae	**pH6-FNA0**	**pH6-FNA0.24**	**pH6-FNA1.09**	**pH6-FNA2.19**	**pH5-FNA0**	**pH5-FNA2.25**	**pH5-FNA13.49**	**pH5-FNA26.98**
TL*	9.01 (±0.6)	13.23(±0.2)	15.58 (±0.4)	18.96 (±0.1)	21.75 (±0.3)	18.77 (±0.5)	23.54 (±0.2)	23.84 (±0.3)	24.35 (±0.3)
FA	4.73	7.22	9.24	12.60	15.62	7.62	13.89	9.63	9.73
SFA	0.96	1.67	2.19	2.47	3.26	1.71	3.14	3.12	3.42
USFA	3.77	5.55	7.05	10.13	12.36	5.91	10.75	6.51	6.31
MUFA	0.25	0.96	0.96	1.31	1.81	0.91	1.64	1.53	1.73
PUFA	3.52	4.59	6.09	8.82	10.55	5.00	9.11	4.98	4.58
CN	44.4	49.9	47.2	47.1	47.0	48.6	48.8	54.3	56.1
DU	153.9	131.1	142.1	145.5	147.0	144.4	143.8	119.4	112.5
C16	0.96	1.67	2.19	2.47	3.26	1.71	3.14	3.12	3.42
C16:1	0.04	0.28	0.23	0.31	0.42	0.15	0.25	0.22	0.31
C16:2	0.00	0.01	0.00	0.00	0.04	0.06	0.08	0.00	0.04
C16:3	0.25	0.21	0.36	0.49	0.58	0.65	1.10	0.75	0.41
C16:4	0.79	0.73	1.26	1.69	1.94	0.92	1.70	0.53	0.51
C18:1	0.15	0.64	0.69	0.94	1.29	0.64	1.05	1.07	1.20
C18:2	0.71	0.91	1.33	1.94	2.35	1.73	3.25	2.29	2.35
C18:3	1.27	1.16	2.04	3.33	3.99	1.17	2.20	1.13	1.15
C20:1	0.00	0.00	0.00	0.00	0.00	0.07	0.17	0.10	0.12
C20:4	0.50	1.57	1.10	1.37	1.65	0.47	0.78	0.28	0.12
C22:1	0.06	0.04	0.04	0.06	0.10	0.05	0.17	0.14	0.10

* Data from Bai *et al.* (2014).

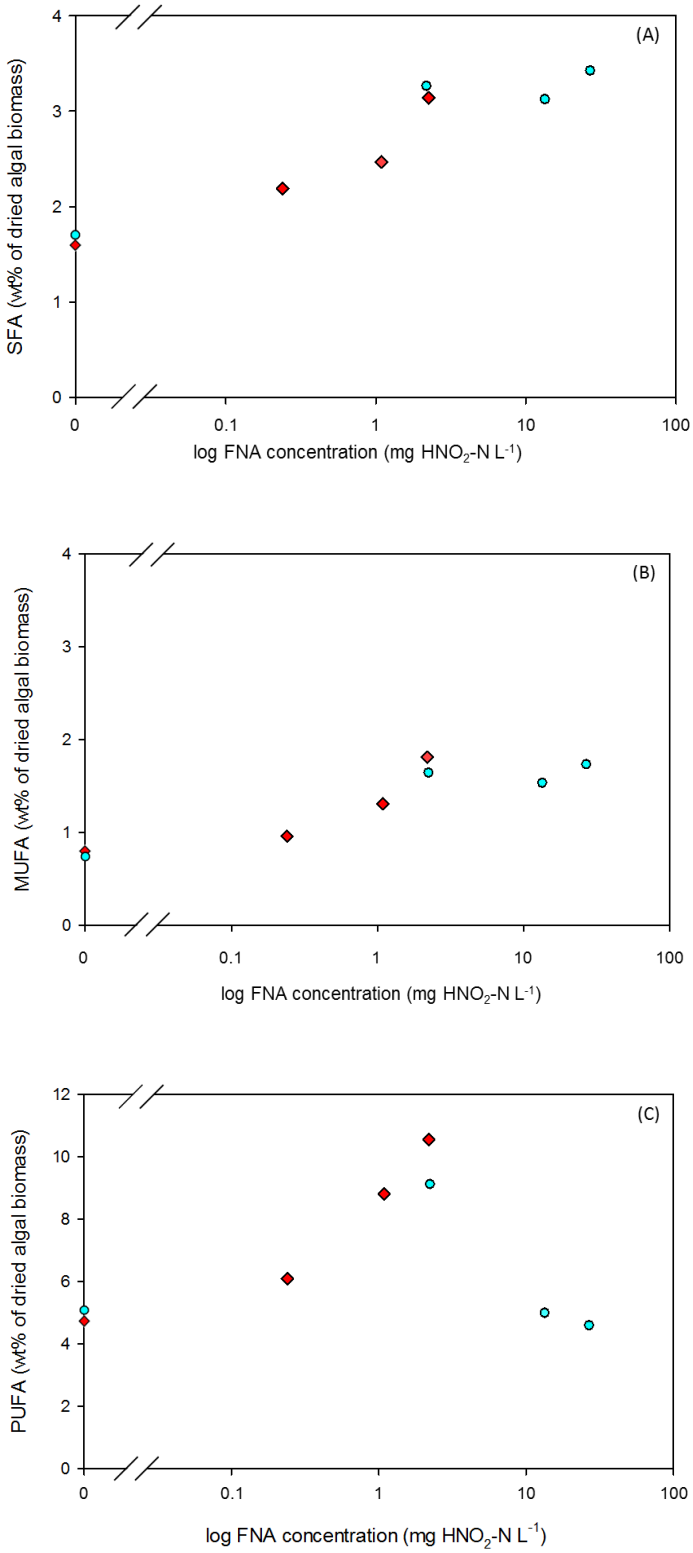


Figure 5.11 Different types of fatty acids recovery after FNA pre-treatment. Red diamonds show results of Batch 1, cyan circles show results of Batch 2.

5.1.3 Research objective 3 (RO3) – Enhancing methane production from algal digestion by free nitrous acid pre-treatment

xv. FNA pre-treatment effect on anaerobic digestion

The cell envelope of algae is described as a rigid barrier and therefore limits access for biological activity in algal anaerobic digestion which is one the main reasons for the relatively low methane yield. In this thesis, the gain in methane yield and production rate is quantified and addressed for the first time by applying FNA pre-treatment to algae.

Kinetic analysis was applied to help understand the effect of FNA pre-treatment on the algae digestion (Section 4.1.6). Algae apparently consists of rapidly and slowly degradable components, with slowly degradable components including polymers and structurally complex parts of the cells (Bohutskyi et al., 2014). The hydrolysis of these slowly degradable components is the rate-limiting step of anaerobic digestion (Eastman & Ferguson, 1981). Therefore, a two-substrate model was applied in this study. The simulated methane production curves using the two-substrate model are shown in Figure 5.12, along with cumulative methane produced in the BMP test. The figure shows methane production was well predicted by the two-substrate model.

The biochemical methane potentials ($B_{0,rapid}$ and $B_{0,slow}$) and reaction rates (k_{rapid} and k_{slow}) are shown in Table 5.6. Comparing BMP1 to BMP-control shows that FNA2.31 pre-treatment dramatically enhanced the methane potential with an increase in both in $B_{0,rapid}$ and $B_{0,slow}$ observed. With 2.31FNA pre-treatment, $B_{0,rapid}$ and $B_{0,slow}$ increased to 0.40 and 0.43 g CH_4-COD per g COD added compared to 0.30 and 0.22 g CH_4-COD per g COD added in the case of untreated algae (BMP-control).

Comparing BMP2-4 to BMP-control shows that higher FNA concentrations were counterproductive for digestion of algae, with methane potentials ($B_{0,total}$) for the high FNA pre-treatments being less than for the BMP control.

In contrast to ultimate biodegradability, the reaction rates k_{rapid} and k_{slow} both decreased after FNA pre-treatment, likely due to multiple factors. The k_{slow} and k_{rapid} in the two-substrate model are lumped parameters, affected by substrate availability, inhibition, physical access to substrate, and even live cell counts. The factors contributed to reduced k_{slow} and k_{rapid} for the FNA systems in most cases, particularly for k_{rapid}, which reflects the kinetics through the early stages of digestion, when the presence of nitrite would be most significant.

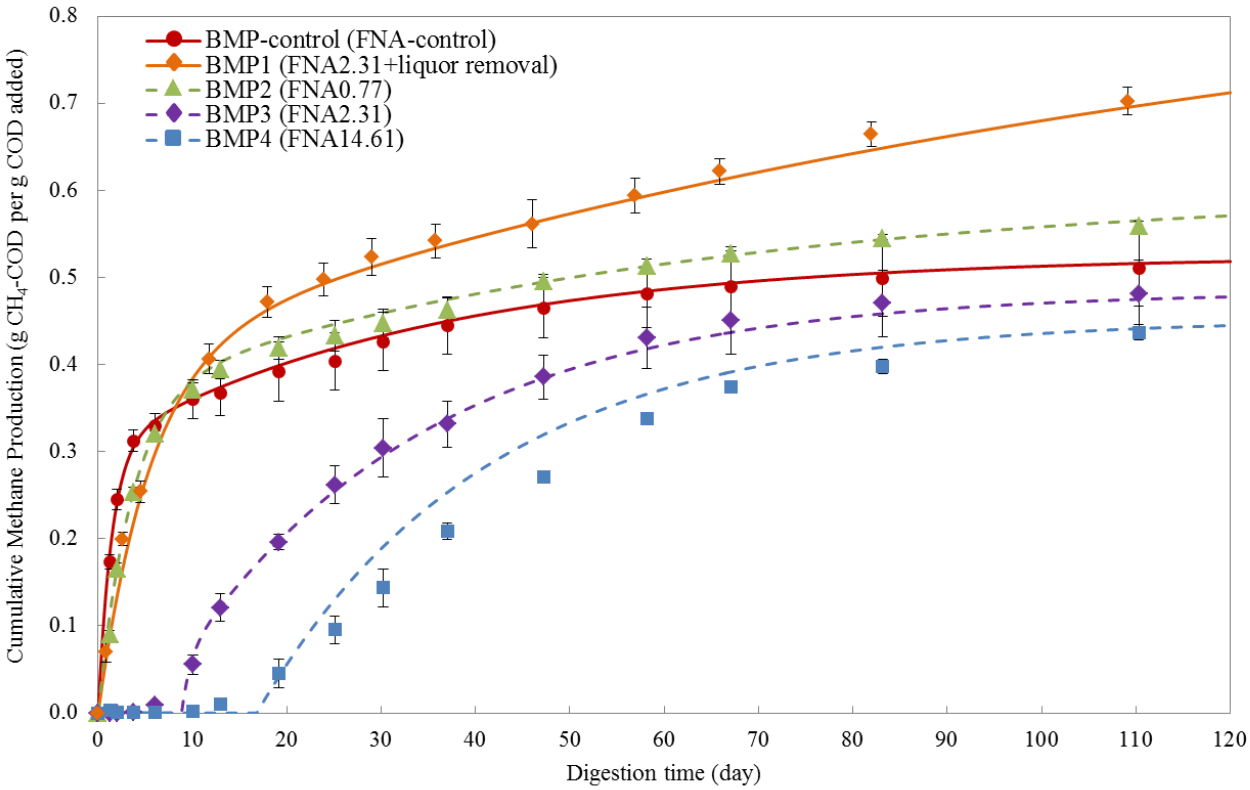


Figure 5.12 Cumulative methane production (g CH_4-COD per g COD added) from algae. Error bars show standard error in triplicate tests and lines show model predicted trends using two-substrate model.

Table 5.6: Estimated k_{rapid}, $B_{0,rapid}$, and k_{slow}, $B_{0,slow}$, t_d using two-substrate model (with 95 % confidence intervals). Calculated $B_{0,total}$ and yields of BMP tests with and without FNA pre-treatment (mean ± SE). SE shows standard errors of triplicate tests.

Sample	k_{rapid} (d^{-1})	$B_{0,rapid}$ (g CH_4-COD per g COD added)	k_{slow} (d^{-1})	$B_{0,slow}$ (g CH_4-COD per g COD added)	t_d	Anaerobic digestion yield (Y_{AD})			Overall yield (Y_O)		
						COD basis (g CH_4-COD per g COD added to AD)	VS basis (L CH_4 per kg VS added to AD)	Increase /Decrease [a] (%)	COD basis (g CH_4-COD per g TCOD added)	VS basis (L CH_4 per kg total VS added)	Increase /Decrease [a] (%)
BMP-control	0.70±0.08	0.30±0.01	0.026±0.01	0.22±0.01	-	0.53±0.01	161±7	-	0.53±0.01	161±7	-
BMP1	0.22±0.03	0.40±0.02	0.012±0.002	0.43±0.02	-	0.82±0.02	254±1	55	0.80±0.02	250±2	53
BMP2	0.29±0.02	0.25±0.03	0.019±0.04	0.25±0.01	-	0.50±0.03					
BMP3	0.14±0.02	0.086±0.03	0.034±0.002	0.42±0.03	8.9±2	0.51±0.03					
BMP4	0.10±0.02	0.023±0.01	0.039±0.006	0.44±0.01	17±1	0.46±0.01					

[a] compared to the BMP-control, on COD basis.

xvi. FNA pre-treatment enhanced methane yield

In order to evaluate the effect of FNA pre-treatment on the methane yield from anaerobic digestion, two yields were determined from the experiments, namely the anaerobic digestion yield (Y_{AD}) and the overall yield (Y_O), as shown in Figure 5.13. Y_{AD} is methane produced per mass of substrate introduced into the digester. It represents the yield of the anaerobic digestion process. Y_O is the methane produced per mass of substrate introduced into the system (before FNA pre-treatment), and so represents the yield of the overall process.

As shown in Table 5.6, the overall methane yields from untreated algae (BMP-control) and FNA pre-treated algae (BMP1) observed in this study are relatively low when compared to those reported on *Tetraselmis* sp. in other studies, ranging between 100 and 400 L CH_4 per kg VS added (Bohutskyi et al., 2014; Marzano et al., 1982). This was expected as the algal biomass was not enriched for lipids (which resulted in low C/N ratio in algal biomass) and the algae had a high culture age (retention time of 15 days). Both these factors have been known to decrease the methane yields (Uggetti et al., 2014; Venkata Mohan et al., 2008).

Comparing BMP1 to BMP-control, the FNA2.31 pre-treatment increased the methane yield dramatically, with a 55 % increase in Y_{AD} and a 53 % increase in Y_O. This could be mainly due to the cell disruption by FNA pre-treatment (see Section 5.1.4).

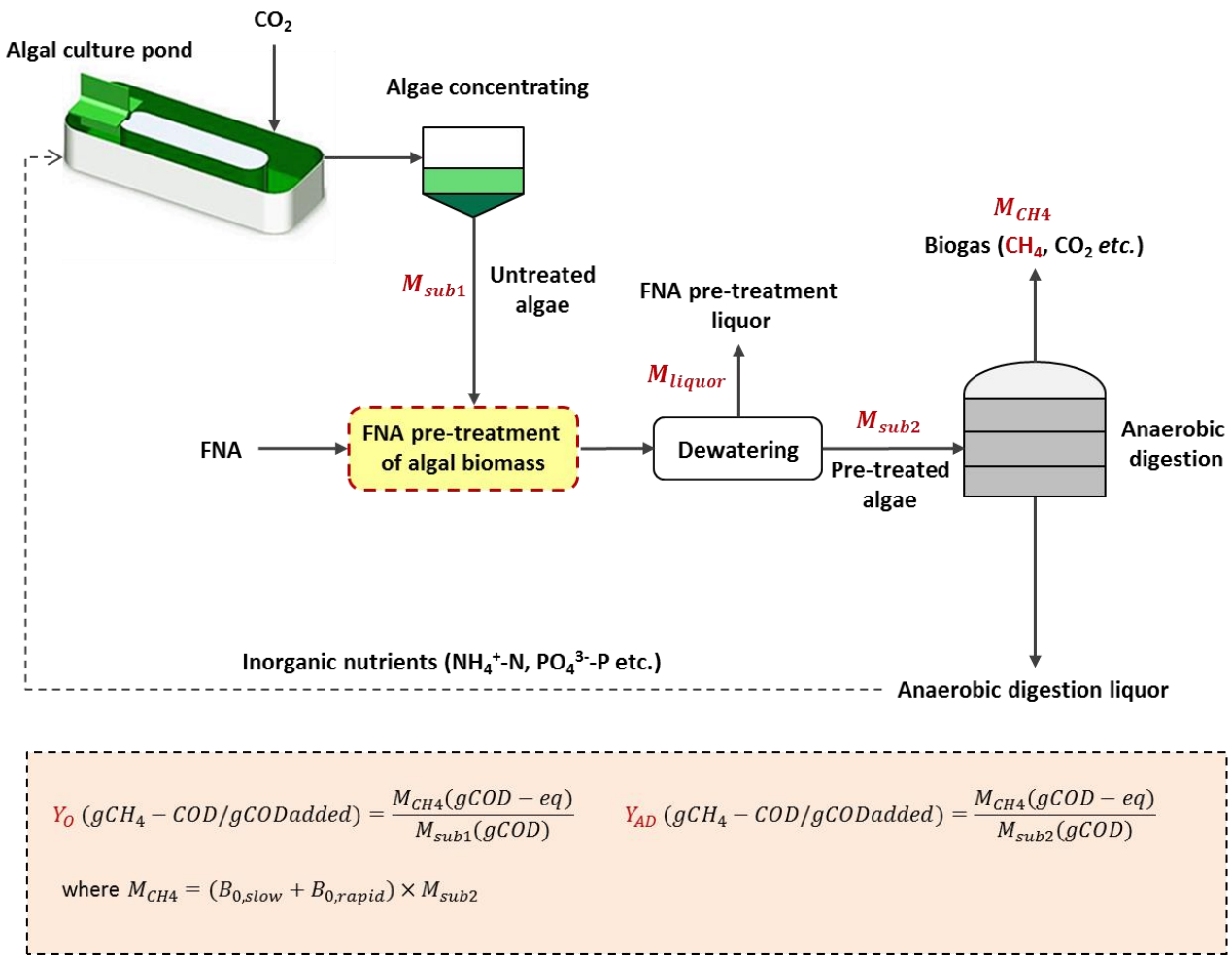


Figure 5.13 Proposed FNA pre-treatment technology for enhancing methane production from algae.

xvii. Contribution of FNA pre-treatment liquor

Including the FNA pre-treatment liquor with the substrate needs careful consideration because the liquor has both positive and negative effects on the methane production. After FNA pre-treatment, the composition of the solubilised organic matter in the pre-treatment liquor was mainly determined as soluble protein and polysaccharides (see Section 5.1.4, Figure 5.18). These compounds are beneficial to methane production in anaerobic digestion since they are rapidly biodegradable components which can improve the methane production (Wang et al., 2013).

But because of the negative effects of having nitrite in the FNA pre-treatment liquor, carrying pre-treatment liquor into the digestion did not enhance the methane yield. Rather, the overall methane yields decreased 4-13 % for BMP2-4 compared to the BMP-control. Two of these negative effects are: (i) denitrification of NO_2^- and (ii) toxicity of FNA to methanogens.

It is known that NO_2^- is a preferred electron acceptor (Banihani et al., 2009). Table 5.7 shows the

COD requirements for nitrite reduction and growth of denitrifiers. These COD requirements are one of the causes for the obvious delay of methane production in BMP3 and BMP4.

Table 5.7: COD requirements for nitrite reduction (through denitrification) and growth of denitrifiers for nitrite-amended culture used in this study.

BMP	**Nitrite concentration (mg N L^{-1})**	**COD required (mg COD L^{-1})**		
		Denitrification (mg COD L^{-1} [a])	Growth of denitrifiers (mg COD L^{-1} [b])	Total (mg COD L^{-1})
BMP2	53	91	176	267
BMP3	159	273	529	802
BMP4	1006	1724	3350	5074

[a] Calculated based on 1.714 mg COD mg NO_2-N^{-1};

[b] Calculated based on theoretical f_e and f_s values, which were 0.34 and 0.66, respectively, neglecting microbial decay. f_e and f_s are the fractions of the electron donor (electron equivalent basis) used for energy generation (i.e., nitrite reduction) and microbial growth, respectively (Tugtas & Pavlostathis, 2007).

The other drawback of carrying pre-treatment liquor into the digester is that nitrite present in the pre-treatment liquor has potential toxicity to methanogenesis (Banihani et al., 2009; Chen et al., 2008). The effect of nitrite on the inoculum is shown in Figure 5.14. Methane production from BLK-II (lower nitrite concentration) was slightly delayed compared to BLK-I (no nitrite); methane production from BLK-III (upper nitrite concentration) was completely inhibited through the entire BMP tests. Banihani *et al.* (2009) revealed that 95 % or greater inhibition of methanogenesis was evident at the lowest concentrations of added NO_2^- tested (8 mg N L^{-1}); and the recovery was only partial at high NO_2^- concentrations (62-153 mg N L^{-1}). The inhibition/toxic effect of NO_2^- was severe in our study which is in line with other reported works (Balderston & Payne, 1976; Tugtas & Pavlostathis, 2007). Based on the study of Banihani et al. (2009), methanogenesis was inhibited completely in the present of NO_2^- (8-153 mg N L^{-1}) ; but it started once the NO_2^- was denitrified.

The negative effects of nitrite need to be taken into account when considering the kinetic data. The k_{slow} and k_{rapid} in the two-substrate model are lumped parameters, affected by substrate availability, inhibition, physical access to substrate, and even cell counts. As already mentioned, the factors contributed to reduced k_{slow} and k_{rapid} for the FNA systems in most cases, particularly for k_{rapid}, which reflects the kinetics through the early stages of digestion, when the presence of nitrite would be most significant.

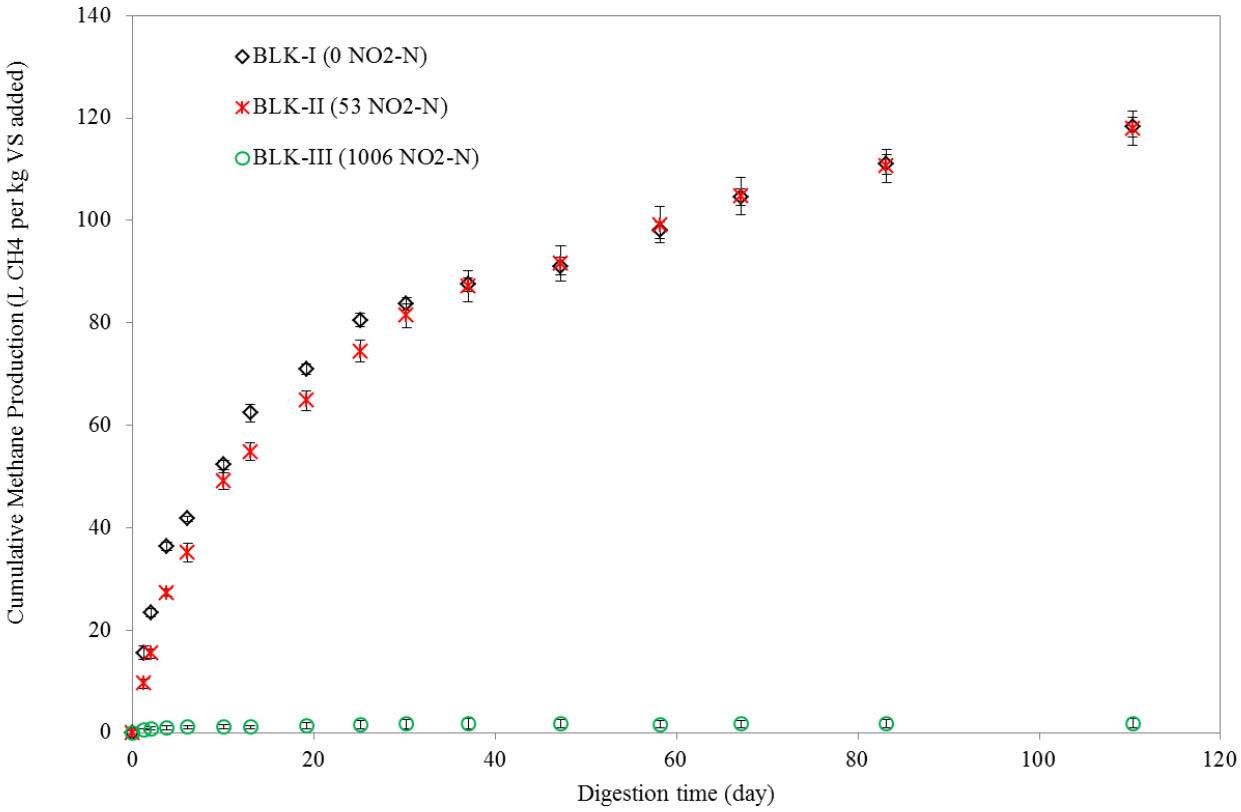


Figure 5.14 Measured methane production from inoculum in BLK-I , II, and III.

5.1.4 Mechanism of downstream process enhancement by FNA pre-treatment

The key outcomes from Research Objective 2 and 3 are that FNA pre-treatment can enhance lipid/TAG recovery efficiency by solvent extraction, and methane yield (both $B_{0,rapid}$ and $B_{0,slow}$) from algae digestion. The contribution of FNA pre-treatment on the enhancement of both downstream processes can be explained as follows:

Untreated algae (as shown in Figure 5.15) pose a rigid cell envelope (cell wall and cell membrane) which limits both lipid extraction yield and rate (lipid extraction), and the methane yield (anaerobic digestion). As shown in the figure, disruption effect is the major contribution by FNA pre-treatment for process enhancement. This effect increases the accessibility of either organic solvent (during lipid extraction) or microorganisms (during anaerobic digestion) to penetrate into algal cells which therefore dramatically improves process performance. The following section outlines the fluorescent microscope analysis, SEM observation, and intracellular compounds release/solubilisation which all provide evidence for the proposed pre-treatment mechanism.

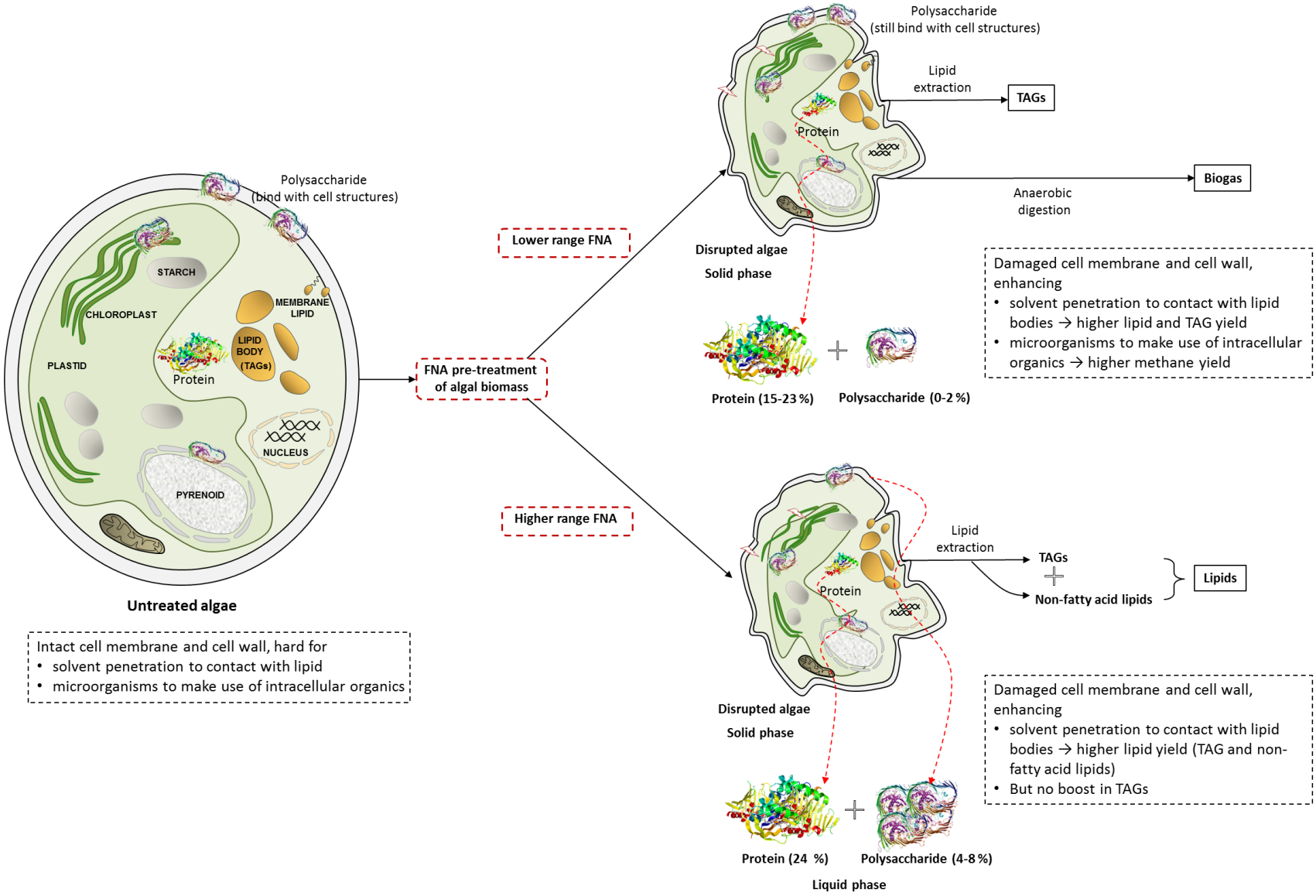


Figure 5.15 Proposed mechanism on downstream process enhancement by FNA pre-treatment.

xviii. Lipid/TAG recovery

As described by Halim *et al.* (2012), the main barrier for TAG (presented in lipid body) extraction is the cell envelope which prohibits organic solvent penetration into the cytoplasm. From this thesis (Section 5.1.2), it is revealed that the main reason for the increased lipid/TAG extraction yield by FNA pretreatment was the degradation of cell membrane and cell walls. This was achieved without associated lipid release (verified by Nile Red staining).

Table 5.8 compares the pretreatment results of two batches of FNA pre-treatment. When the FNA concentration reached 1.09 mg HNO_2-N L^{-1}, the colour of the algal liquor turned to yellowish-green from dark green (natural colour) after the pre-treatment. With a further increase in FNA concentration to 26.98 mg HNO_2-N L^{-1}, the colour turned to brown, indicating denaturation of chlorophyll pigments in the algal cells. This result is consistent with the microscope observations (Figure 5.16). Under the fluorescent microscope, chlorophyll fluoresced as red colour. With increase FNA concentration, membrane damaged cell increased (Table 5.8). Moreover, seldom red autofluorescence was observed in higher FNA trials (pH5-FNA13.49 and pH5-FNA26.98) (Figure 5.16g) after the pretreatment which indicates a severe effect by FNA pre-treatment.

Table 5.8 Outputs culture conditions and membrane damaged cell increase after FNA pre-treatment.

Test	**Batch1**				**Batch 2**			
	pH6 -FNA0	pH6 -FNA0.24	pH6 -FNA 1.09	pH6 -FNA 2.19	pH5 -FNA0	pH5 -FNA2.25	pH5 -FNA13.49	pH5 -FNA26.98
culture appearance after pre-treatment	dark green	dark green	yellowish-green	yellowish-green	dark green	yellowish-green	brown	brown
membrane damaged cell increase (%)	69	72	80	92	85	96	96	96

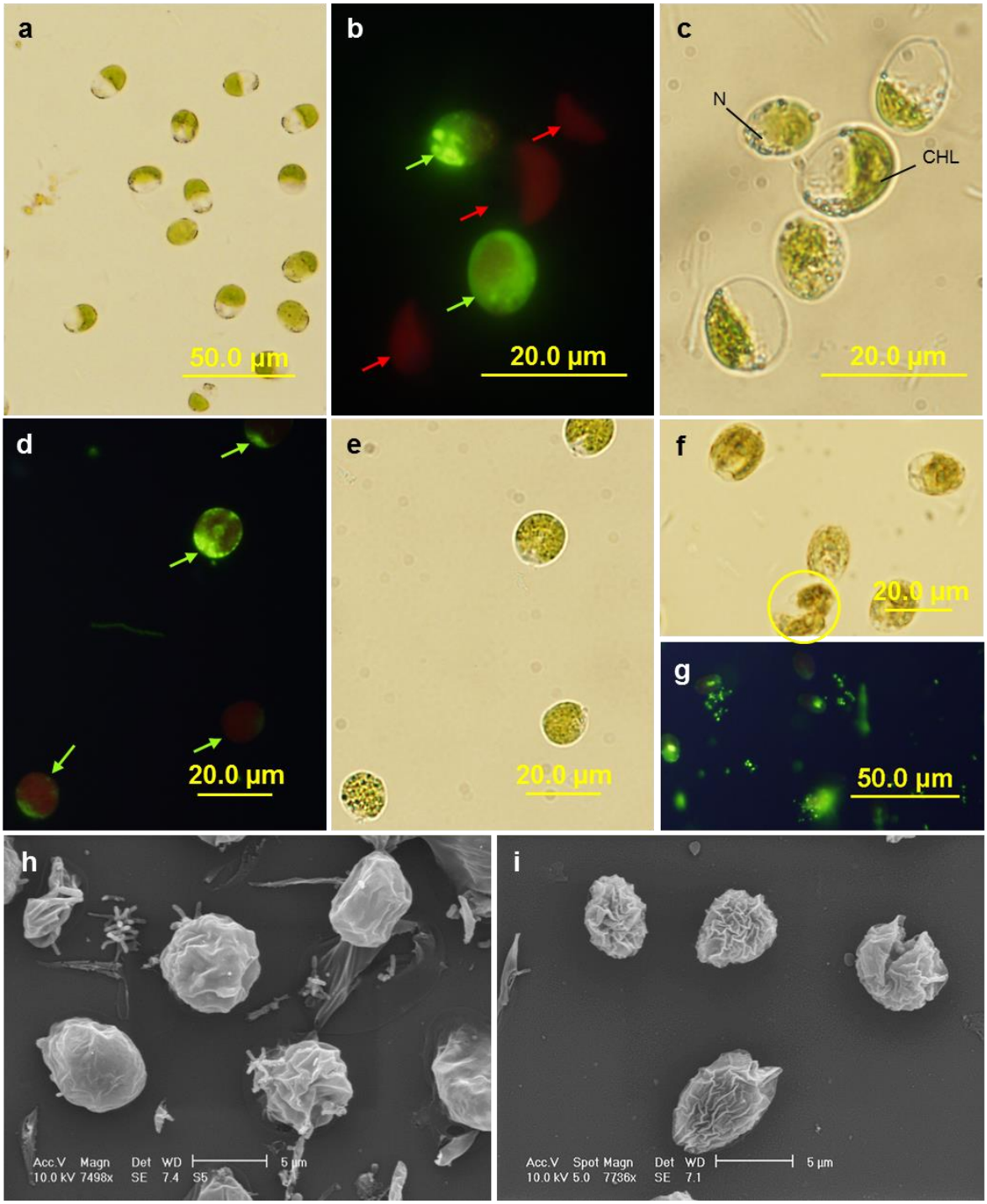


Figure 5.16 Light microscope and SEM images of untreated and FNA-treated cells under different conditions. (a) untreated cells. (b) pH6-FNA0 algal cells after 48 h pretreatment. (c) fluorescent image of the same field of image b. (d) pH6-FNA2.19 algal cells after 48 h pretreatment. (e) fluorescent image of the same field of image d. (f) pH5-FNA26.98 algal cells after 48 h pretreatment. (g) fluorescent image of pH5-FNA26.98. (h) and (i) SEM images of untreated cells and pH6-FNA2.19 algal cells after 48 h pretreatment. Green arrows show membrane damaged cells; Red arrows show intact cells. Yellow circle shows cell fragmentation. N: Nucleus. CHL: Chloroplast.

In this study, cell disruption was characterised by protein and polysaccharides release (Figure 5.17), and improved access to TAG. But significantly, Figure 5.17 shows the release of protein and polysaccharides as a function of FNA concentration was markedly different, with protein being released after exposure to low FNA concentration but polysaccharides mainly being released after exposure to high FNA concentration. For single-cell green algae, such as *Tetraselmis* sp. applied in the study, most of the protein content of the biomass belongs to the intracellular components, whereas polysaccharides are the main constituent of the cell wall and intracellular starch components (such as storage starch granules and shell starch grains surrounding pyrenoids) (Gibbs, 1962). Exposure to low FNA concentration apparently led to minor cell disruption, sufficient to release protein and improve access to TAG. Exposure to high FNA concentration apparently led to deep penetration into chloroplasts and membrane lipids (i.e. phospholipids), characterised by relatively high polysaccharide mobilisation and potentially elevated recovery of non-TAG lipids, which was countered by oxidation of some TAG.

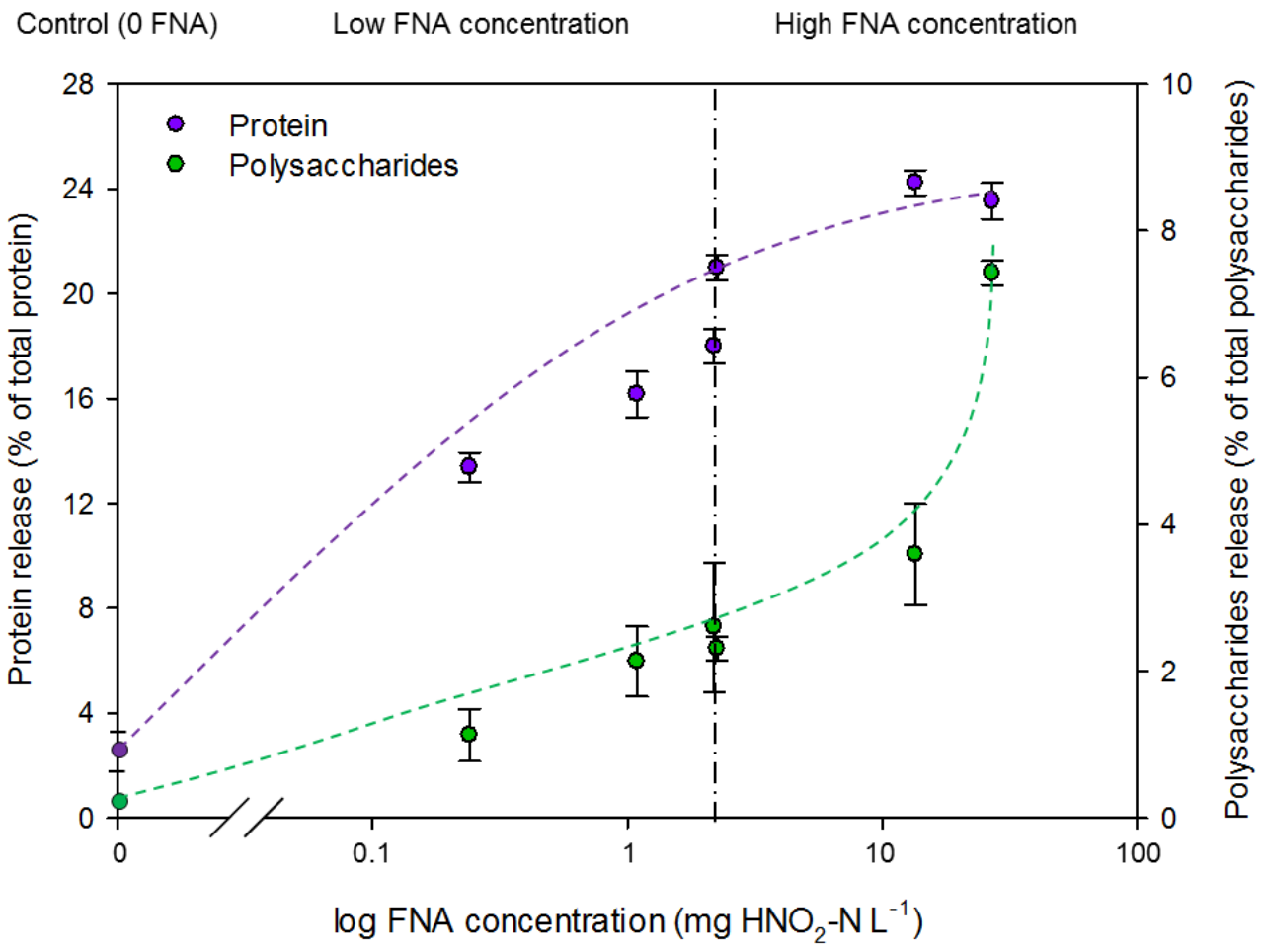


Figure 5.17 Protein and polysaccharides recovery released from algal cells through pretreatment.

xix. Methane yield in AD

The BMP tests showed that FNA pre-treatment can result in a boost in both $B_{0,rapid}$ and $B_{0,slow}$, and consequently a boost in Y_{AD} (Section 5.1.3). This is likely due to the pre-treatment disrupting cell envelope and even intracellular membranes. Both can act as barriers to methanogenic activity on encapsulated organics.

Disruption of the algal cells is revealed and described above by the fluorescent microscope assay. Disrupting cells led to a boost in soluble substrates, likely due to release of organics. Figure 5.18A shows the increase in SCOD, soluble protein and polysaccharides concentration after the FNA2.31 pre-treatment, compared to the changes from BMP-control. As shown in Figure 5.18A, solubilisation of organic matter (SCOD) increased 5.7 times more after FNA2.31 pre-treatment compared to the control. The characterisation of the soluble organic matter shows that the SCOD mainly consisted of protein and polysaccharides. After algae was treated at an FNA level of 2.31 mg HNO_2-N L^{-1}, soluble protein and polysaccharides concentrations in the pre-treatment liquor increased from 10.2 to 110 g kg VS^{-1} and 10.1 to 20.5 g kg VS^{-1}, respectively. Additionally, released protein was not degraded to NH_4-N during the pre-treatment (Figure 5.18B), which benefits the digestion process.

But the boost in soluble substrates is far outweighed by the increase in $B_{0,slow}$. The increase in $B_{0,slow}$ is a unique and important result. Considering FNA pre-treatment of waste activated sludge (WAS), availability of slowly biodegradable substrates seemed to be unaffected by FNA pre-treatment (Wang et al., 2013). But the results of Research Objective 3 show that for algae, $B_{0,slow}$ can be doubled with FNA pre-treatment (BMP1 compared to BMP-control). For algal cells, the rigid cell envelope and even intracellular structures are major barriers to effective digestion (Bohutskyi et al., 2014).

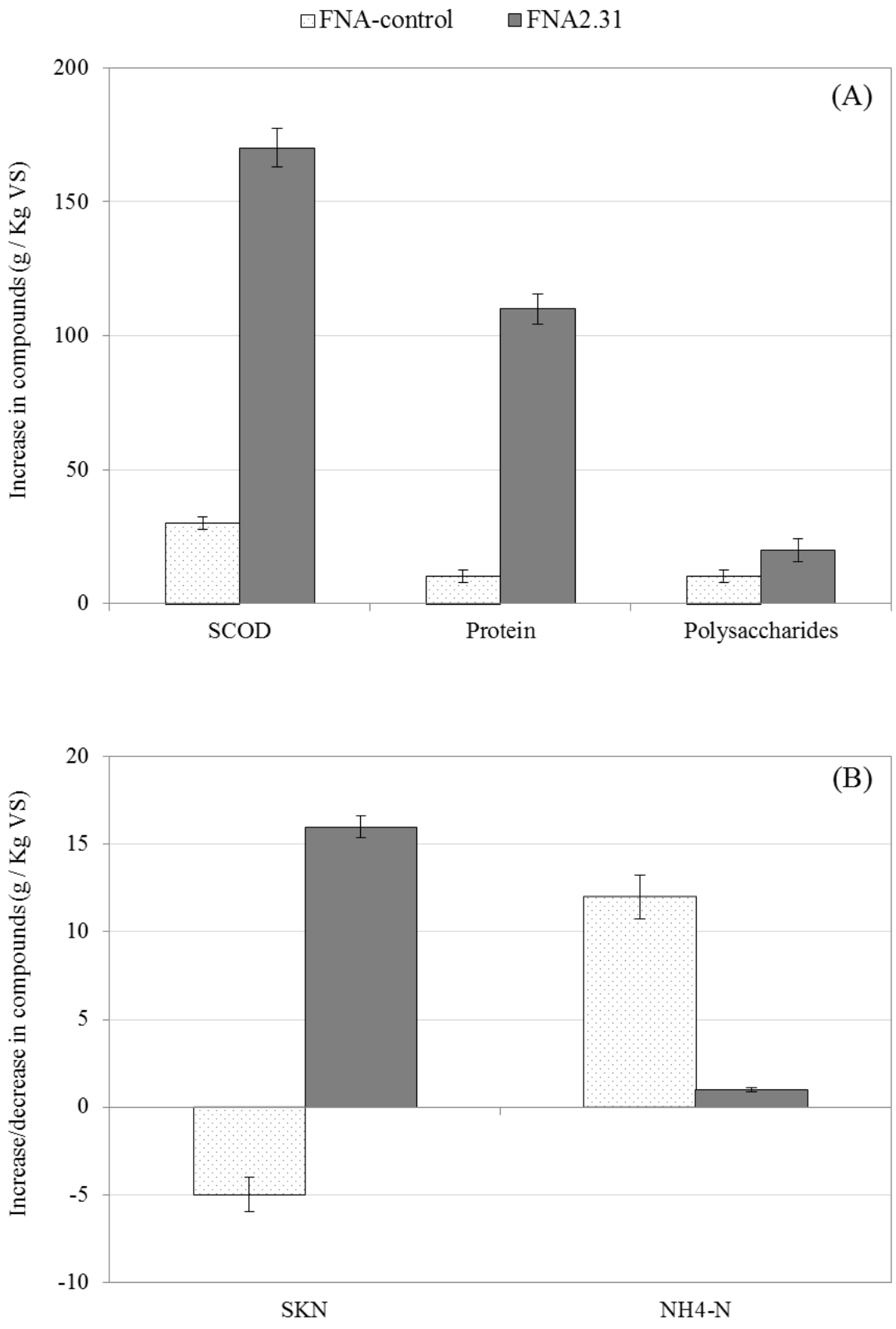


Figure 5.18 FNA pre-treatment on algal biomass solubilisation in terms of (A) SCOD, protein and polysaccharides concentration and (B) SKN-N and NH_4^+-N.

6 CONCLUSIONS AND RECOMMENDATIONS FOR FUTURE RESEARCH

6.1 Conclusions, outcomes and contributions

This research focused on two aspects of the algal biofuel pipeline: the upstream algal cultivation, and cell pre-treatment to enhance downstream processing. The first research objective was to understand the contribution of heterotrophic bacteria to the growth of algae, following with the development of an integrated model for open algal culture systems. The second and third research objectives focused on downstream process improvement by free nitrous acid (FNA) pre-treatment.

The outcome from the first part of the work is the understanding of the contribution of heterotrophic bacteria to the growth of algae. It was found that adding heterotrophic bacteria to an open algal culture dramatically enhances the growth of algae under two different pH values. This was due to bacteria cycling carbon by re-mineralising photosynthetic end products. In this work, the contribution of bacteria was modelled through a range of pH values and it was shown that supplementing bacteria to an open algal system can overcome the inorganic carbon limitation that would be expected in an air supplied algal culture.

In addition, an integrated Algae-Bacteria Model (ABM) was developed and presented in a Petersen matrix format. This model provides the first description of algal cultivation processes considering the effect of heterotrophic bacteria on carbon cycling and oxygen consumption.

The second part of the thesis focused on downstream process enhancement by free nitrous acid (FNA) pre-treatment. Key conclusions are:

(1) Total lipid including triacylglyceride recovery was enhanced by FNA pre-treatment.
- The total lipid extraction yield from algae was found to be enhanced with higher FNA concentration (up to 2.19 mg HNO_2-N L^{-1}) and longer pretreatment time (48 h).
- The highest total lipid extraction yield was found to be 21.9 ± 0.2 % (wt % of dried algal biomass), which was 2.4-fold higher compared to that from untreated algae.
- The mass transfer coefficient (k) for lipid extraction using hexane from algae treated with 2.19 mg HNO_2-N L^{-1} FNA was found to increase dramatically.
- In general, the quality of the biodiesel that could be obtained was improved by FNA pre-treatment.

(2) Methane yield from algal digestion was enhanced by FNA pre-treatment.

- The methane production yield from algae through anaerobic digestion was found to be dramatically enhanced by FNA pre-treatment (2.31 mg HNO_2-N L^{-1}), with a 55 % increase in Y_{AD} and a 53 % increase in Y_O.
- Detailed kinetic analysis of methane production from BMP tests based on model simulation indicates that with FNA2.31 pre-treatment, $B_{0,slow}$ and $B_{0,rapid}$ increased to 0.40 and 0.43 g CH4-COD per g COD added comparing to 0.30 and 0.22 g CH4-COD per g COD added in the case of untreated algae (BMP-control).
- FNA pre-treatment would be an efficient method for enhancing methane production from algal biomass through anaerobic digestion.

(3) FNA pre-treatment acts via physical cell disruption.

- Cell membrane damage assays and SEM observations revealed the cell envelope disruption effect by FNA pre-treatment. This was the main reason for the enhanced lipid/TAG recovery and methane yield in algal digestion.
- Investigation of the release of intracellular components revealed that low FNA concentration led to minor cell disruption, sufficient to release protein and improve access to TAG. However, high FNA concentration apparently led to deep penetration into chloroplasts and membrane lipids (i.e. phospholipids), which potentially elevated recovery of non-TAG lipids, which was countered by oxidation of some TAG. This explains why the optimized FNA concentration for TAG recovery was around 2 mg HNO_2-N L^{-1}. Higher FNA concentration was not effective for TAG recovery.
- The disruption of the cell envelope and intracellular membrane contribute to the enhanced ultimate methane potential $B_{0,slow}$ and $B_{0,rapid}$ in anaerobic digestion.

6.2 Recommendations for future research

Regarding the activity of heterotrophic bacteria and modelling of the open algal cultivation systems, several questions arise which will be interesting to address:

- It has been demonstrated both by experiments and modelling that heterotrophic bacteria can both recycle carbon (Bai et al., submitted 2014) and consume oxygen (Buhr & Miller, 1983; Park & Craggs, 2010) in the open algal culture systems. It is also known that decreasing DO level and/or increasing carbon dioxide concentration available for algal cells can stimulate lipid synthesis in the cells (Chiu et al., 2009; Choi et al., 1982; Hu & Gao, 2006). But the effect of heterotrophic bacteria on algal lipid and carbon hydrate accumulation has not yet been reported. Furthermore, adding bacterial biomass into algal culture generates mixed biomass which will be processed in downstream steps such as biodiesel production. The effect of bacteria supplementation to biofuel production as mixed biomass also needs consideration. These are of particular interest when considering the use of algae for the generation of biodiesel as lipid represent potential biodiesel source. Knowledge on the role of carbon cycling or oxygen consumption by heterotrophic bacteria in the accumulation of lipid in algae will assist in determining the optimal operating conditions for the production of algal biodiesel.

- The established algae-based model, considering the activity of bacteria, has potential to predict algal productivity and process performance (Bai et al., submitted 2014). However, for the purpose of biofuel production from algae, modelling and controlling the intracellular compounds production (such as lipids and carbohydrates) is required. Up to now, only two models are available to predict neutral lipid synthesis in algae as function of nitrogen source and/or available light (Mairet et al., 2011; Packer et al., 2011). However, both models failed to consider the existence of bacteria, which results in the difficulty to use in the open algae cultivation systems. Therefore, it is interesting to be able to integrate the lipid accumulation processes in the Algae-bacteria model which can offer a convenient tool for lipid production prediction and control in open algae cultivation systems.

Regarding the use of FNA pre-treatment as a technology to enhance downstream process from algae, several questions arise which will be useful to address for industrial application (Figure 6.1):

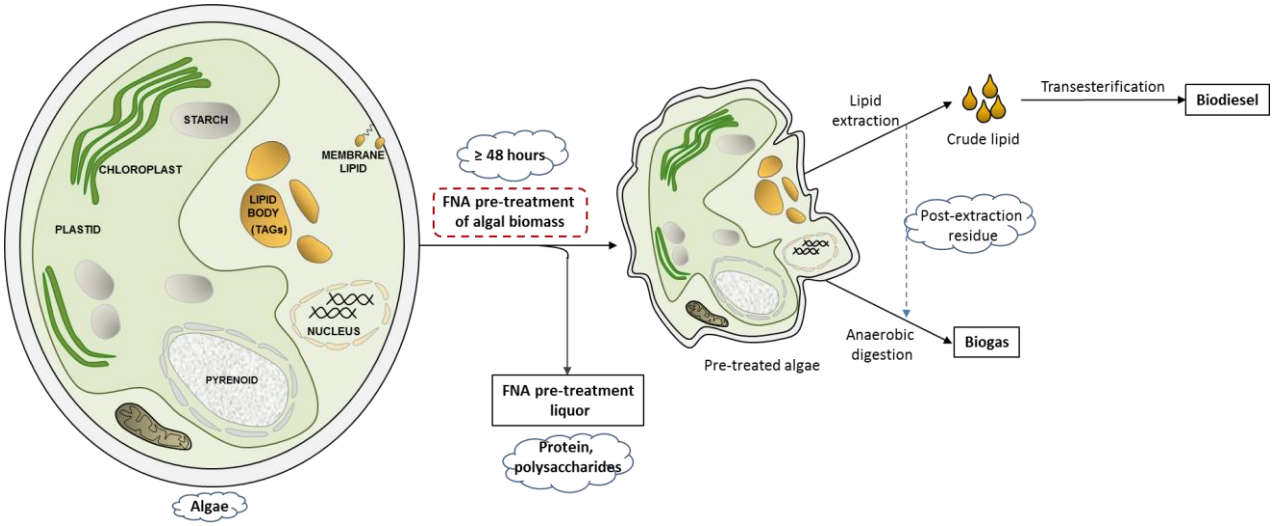


Figure 6.1 Current FNA pre-treatment technology and recommendations for further research.

- In this thesis, the effect of FNA pre-treatment and optimal FNA concentration for lipid/TAG recovery was tested on *Tetraselmis* sp. However, pre-treatment technologies generally have different effects on different types of algae due to the varying cell ultrastructures and physical resistance of algae. For example, in the study operated by Prabakaran and Ravindran (2011), osmotic shock pre-treatment method was more effective on *Tolypothrix* sp. than on *Nostoc* sp, with an increase on lipid extraction yield of 3 times and 1.5 time, respectively. Thus, determination of the optimum concentration of FNA pre-treatment depending on algal species is beneficial for large scale application, especially for mixed algae.

- The effective cell disruption by FNA pre-treatment in this thesis was revealed to be more than 48 hours of contact time (Bai et al., 2014). As reported by Wang et al. (2013), the effective pre-treatment contact time was shown to be 24 hours or less. Ideally, good mixing is required during the entire pre-treatment to provide a homogeneous contact of algae to the FNA. Thus, it would be beneficial, from the energy and cost perspective, to decrease the pre-treatment contact time.

- A consequence of FNA pre-treatment, some protein and polysaccharides were released from the algal cells in the pre-treatment liquor. Some algal protein and polysaccharides are 10-500 times higher value products than biodiesel (Becker, 2006; Fernández-Reiriz et al., 1989). It

would be interesting to characterise the protein and polysaccharides recovered after FNA pre-treatment.

Reference

Alabi, A.O., Bibeau, E., Tampier, M., Council, B.C.I. 2009. Microalgae technologies & processes for biofuels-bioenergy production in British Columbia: Current technology, suitability & barriers to implementation: final report. British Columbia Innovation Council.

Alzate, M.E., Muñoz, R., Rogalla, F., Fdz-Polanco, F., Pérez-Elvira, S.I. 2014. Biochemical methane potential of microalgae biomass after lipid extraction. Chemical Engineering Journal, 243(1), 405-410.

American Public Health Association. 2005. Standard methods for the examination of water and wastewater. APHA-AWWA-WEF, Washington, DC.

Amin, S. 2009. Review on biofuel oil and gas production processes from microalgae. Energy Conversion and Management, 50(7), 1834-1840.

Andersen, R.A. 2005. Algal culturing techniques. Elsevier/Academic Press, Boston, Mass.

Anthonisen, A.C., Loehr, R.C., Prakasam, T.B.S., Srinath, E.G. 1976. Inhibition of nitrification by ammonia and nitrous acid. Journal (Water Pollution Control Federation), 48(5), 835-852.

Arts, M.T., Ackman, R.G., Holub, B.J. 2001. " Essential fatty acids" in aquatic ecosystems: a crucial link between diet and human health and evolution. Canadian Journal of Fisheries and Aquatic Sciences, 58(1), 122-137.

Ask, J., Karlsson, J., Persson, L., Ask, P., Byström, P., Jansson, M. 2009. Whole-lake estimates of carbon flux through algae and bacteria in benthic and pelagic habitats of clear-water lakes. Ecology, 90(7), 1923-1932.

Azov, Y., Goldman, J.C. 1982. Free ammonia inhibition of algal photosynthesis in intensive cultures. Applied and environmental microbiology, 43(4), 735-739.

Azov, Y., Shelef, G., Moraine, R. 1982. Carbon limitation of biomass production in high-rate oxidation ponds. Biotechnology and bioengineering, 24(3), 579-594.

Bai, X., Ghasemi Naghdi, F., Ye, L., Lant, P., Pratt, S. 2014. Enhanced lipid extraction from algae using free nitrous acid pretreatment. Bioresource Technology, 159, 36-40.

Baisuo, Z., Jingwei, M., Quanbao, Z., Craig, F. 2009. Anaerobic digestion of algal biomass residues with nutrient recycle. Washingon State University.

Balderston, W.L., Payne, W.J. 1976. Inhibition of methanogenesis in salt marsh sediments and whole-cell suspensions of methanogenic bacteria by nitrogen oxides. Applied and Environmental Microbiology, 32(2), 264-269.

Banga, J.R., Bogaerts, P., Van Impe, J., Dochain, D., Smets, I. 2010. Hurdles and challenges for modelling and control of microalgae for CO2 mitigation and biofuel production.

Banihani, Q., Sierra-Alvarez, R., Field, J. 2009. Nitrate and nitrite inhibition of methanogenesis during denitrification in granular biofilms and digested domestic sludges. Biodegradation, 20(6), 801-812.

Baquerisse, D., Nouals, S., Isambert, A., dos Santos, P.F., Durand, G. 1999. Modelling of a continuous pilot photobioreactor for microalgae production. in: Progress in Industrial Microbiology, (Eds.) J.T.J.G.B. R. Osinga, R.H. Wijffels, Vol. Volume 35, Elsevier, pp. 335-342.

Batstone, D.J., Tait, S., Starrenburg, D. 2009. Estimation of hydrolysis parameters in full-scale anerobic digesters. Biotechnology and Bioengineering, 102(5), 1513-1520.

Béchet, Q., Shilton, A., Guieysse, B. 2013. Modeling the effects of light and temperature on algae growth: State of the art and critical assessment for productivity prediction during outdoor cultivation. Biotechnology Advances, 31(8), 1648-1663.

Becker, E.W. 2006. Microalgae as a source of protein. Biotechnology Advances, 25(2), 207-210.

Beer, L.L., Boyd, E.S., Peters, J.W., Posewitz, M.C. 2009. Engineering algae for biohydrogen and biofuel production. Current Opinion in Biotechnology, 20(3), 264-271.

Bell, G. 1982. The masterpiece of nature: the evolution and genetics of sexuality. Springer.

Benson, B.C., Gutierrez-wing, M.T., Rusch, K.A. 2007. The development of a mechanistic model to investigate the impacts of the light dynamics on algal productivity in a Hydraulically Integrated Serial Turbidostat Algal Reactor (HISTAR). 36(2), 14.

Bernard, O., Rémond, B. 2012. Validation of a simple model accounting for light and temperature effect on microalgal growth. Bioresource Technology, 123, 520-527.

Bligh, E., Dyer, W. 1959. A rapid method of total lipid extraction and purification. Can. J. Biochem. Physiol, 37(8), 911-917.

Bloesch, J., Stadelmann, P., Buhrer, H. 1977. Primary production, mineralization, and sedimentation in the euphotic zone of two Swiss lakes. Limnol. Oceanogr, 22(5), 1.

Bohutskyi, P., Betenbaugh, M.J., Bouwer, E.J. 2014. The effects of alternative pretreatment strategies on anaerobic digestion and methane production from different algal strains. Bioresource Technology, 155, 366-372.

Bohutskyi, P., Bouwer, E. 2013. Biogas production from algae and cyanobacteria through anaerobic digestion: a review, analysis, and research needs. in: Advanced Biofuels and Bioproducts, (Ed.) J.W. Lee, Springer New York, pp. 873-975.

Brennan, L., Owende, P. 2010. Biofuels from microalgae-A review of technologies for production, processing, and extractions of biofuels and co-products. Renewable and Sustainable Energy Reviews, 14(2), 557-577.

Buchanan, J.J., Slater, F.R., Bai, X., Pratt, S. 2012. Algal growth and community structure in a mixed-culture system using coal seam gas water as the water source. Environmental Technology, 34(6), 695-701.

Buhr, H.O., Miller, S.B. 1983. A dynamic model of the high-rate algal-bacterial wastewater treatment pond. Water Research, 17(1), 29-37.

Carvalho, A., Silva, S., Baptista, J., Malcata, F. 2011. Light requirements in microalgal photobioreactors: an overview of biophotonic aspects. Applied Microbiology and Biotechnology, 89(5), 1275-1288.

Chen, C.-Y., Yeh, K.-L., Aisyah, R., Lee, D.-J., Chang, J.-S. 2011a. Cultivation, photobioreactor design and harvesting of microalgae for biodiesel production: A critical review. Bioresource Technology, 102(1), 71-81.

Chen, M., Tang, H., Ma, H., Holland, T.C., Ng, K.Y.S., Salley, S.O. 2011b. Effect of nutrients on growth and lipid accumulation in the green algae Dunaliella tertiolecta. Bioresource Technology, 102(2), 1649-1655.

Chen, Y., Cheng, J.J., Creamer, K.S. 2008. Inhibition of anaerobic digestion process: A review. Bioresource Technology, 99(10), 4044-4064.

Cheng, L., Zhang, L., Chen, H., Gao, C. 2006. Carbon dioxide removal from air by microalgae cultured in a membrane-photobioreactor. Separation and Purification Technology, 50(3), 324-329.

Chhetri, A.B., Islam, M.R. 2008. Towards Producing a Truly Green Biodiesel. Energy Sources, Part A: Recovery, Utilization, and Environmental Effects, 30(8), 754-764.

Chisti, Y. 2007. Biodiesel from microalgae. Biotechnology Advances, 25(3), 294-306.

Chisti, Y. 2008. Biodiesel from microalgae beats bioethanol. Trends in biotechnology, 26(3), 126-131.

Chiu, S.-Y., Kao, C.-Y., Tsai, M.-T., Ong, S.-C., Chen, C.-H., Lin, C.-S. 2009. Lipid accumulation and CO2 utilization of Nannochloropsis oculata in response to CO2 aeration. Bioresource Technology, 100(2), 833-838.

Cho, S., Park, S., Seon, J., Yu, J., Lee, T. 2013. Evaluation of thermal, ultrasonic and alkali pretreatments on mixed-microalgal biomass to enhance anaerobic methane production. Bioresource Technology, 143(0), 330-336.

Choi, S., Ryu, D., Rhee, J. 1982. Production of microbial lipid: Effects of growth rate and oxygen on lipid synthesis and fatty acid composition of Rhodotorula gracilis. Biotechnology and Bioengineering, 24(5), 1165-1172.

Christenson, L., Sims, R. 2011. Production and harvesting of microalgae for wastewater treatment, biofuels, and bioproducts. Biotechnology Advances, 29(6), 686-702.

Cohen, Z., Vonshak, A., Richmond, A. 1988. Effect of environmental conditions on fatty acid composition of the red algaa Porphyridium Cruentum: correlation to growth rate. Journal of Phycology, 24(3), 328-332.

Cole, J.J. 1982. Interactions Between Bacteria and Algae in Aquatic Ecosystems. Annual Review of Ecology and Systematics, 13(ArticleType: research-article / Full publication date: 1982 / Copyright © 1982 Annual Reviews), 291-314.

Craggs, R., Heubeck, S., Lundquist, T., Benemann, J. 2011. Algal biofuels from wastewater treatment high rate algal ponds. Water Science & Technology, 63(4), 660-665.

Das, P., Aziz, S.S., Obbard, J.P. 2011. Two phase microalgae growth in the open system for enhanced lipid productivity. Renewable Energy, 36(9), 2524-2528.

de Morais, M.G., Costa, J.A.V. 2007. Isolation and selection of microalgae from coal fired thermoelectric power plant for biofixation of carbon dioxide. Energy Conversion Management, 48(21), 69-73.

Dedon, P.C., Tannenbaum, S.R. 2004. Reactive nitrogen species in the chemical biology of inflammation. Archives of Biochemistry and Biophysics, 423(1), 12-22.

Eastman, J.A., Ferguson, J.F. 1981. Solubilization of particulate organic carbon during the acid phase of anaerobic digestion. Journal (Water Pollution Control Federation), 352-366.

Fajardo, A.R., Cerdán, L.E., Medina, A.R., Fernández, F.G.A., Moreno, P.A.G., Grima, E.M. 2007. Lipid extraction from the microalga Phaeodactylum tricornutum. European Journal of Lipid Science and Technology, 109(2), 120-126.

Fallowfield, H.J., Garrett, M.K. 1985. The treatment of wastes by algal culture. Journal of Applied Microbiology, 59, 187S-205S.

Felizardo, P., Neiva Correia, M.J., Raposo, I., Mendes, J.F., Berkemeier, R., Bordado, J.M. 2006. Production of biodiesel from waste frying oils. Waste Management, 26(5), 487-494.

Fernández-Reiriz, M.J., Perez-Camacho, A., Ferreiro, M.J., Blanco, J., Planas, M., Campos, M.J., Labarta, U. 1989. Biomass production and variation in the biochemical profile (total protein, carbohydrates, RNA, lipids and fatty acids) of seven species of marine microalgae. Aquaculture, 83(1–2), 17-37.

Fukami, K., Nishijima, T., Ishida, Y. 1997. Stimulative and inhibitory effects of bacteria on the growth of microalgae. Hydrobiologia, 358(1), 185-191.

Fukuda, H., Kondo, A., Noda, H. 2001. Biodiesel fuel production by transesterification of oils. Journal of Bioscience and Bioengineering, 92(5), 405-416.

Gallagher, B.J. 2011. The economics of producing biodiesel from algae. Renewable Energy, 36(1), 158-162.

Gibbs, S.P. 1962. The ultrastructure of the pyrenoids of green algae. Journal of Ultrastructure Research, 7(3–4), 262-272.

Goldman, J.C., Graham, S.J. 1981. Inorganic carbon limitation and chemical composition of two freshwater green microalgae. Applied and environmental microbiology, 41(1), 60-70.

Goldman, J.C., Oswald, W.J., Jenkins, D. 1974. The Kinetics of Inorganic Carbon Limited Algal Growth. Journal (Water Pollution Control Federation), 46(3), 554-574.

Golueke, C.G., Oswald, W.J. 1959. Biological Conversion of light energy to the chemical energy of methane. Applied and Environmental Microbiology, 7(4), 219.

González-Fernández, C., Sialve, B., Bernet, N., Steyer, J.-P. 2012. Impact of microalgae characteristics on their conversion to biofuel. Part II: Focus on biomethane production. Biofuels, Bioproducts and Biorefining, 6(2), 205-218.

Grant, M.A., Kazamia, E., Cicuta, P., Smith, A.G. 2014. Direct exchange of vitamin B12 is demonstrated by modelling the growth dynamics of algal–bacterial cocultures. The ISME journal.

Grima, E.M., Camacho, F.G., Pérez, J.A.S., Sevilla, J.M.F., Fernández, F.G.A., Gómez, A.C. 1994. A mathematical model of microalgal growth in light-limited chemostat culture. Journal of Chemical Technology & Biotechnology, 61(2), 167-173.

Grima, E.M., Fernández, F.G.A., García Camacho, F., Chisti, Y. 1999. Photobioreactors: light regime, mass transfer, and scaleup. Journal of Biotechnology, 70(1-3), 231-247.

Gujer, W., Henze, M., Mino, T., Matsuo, T., Wentzel, M.C., Marais, G.v.R. 1995. The Activated Sludge Model No. 2: Biological phosphorus removal. Water science and technology, 31(2), 1-11.

Haas, M.J. 2005. Improving the economics of biodiesel production through the use of low value lipids as feedstocks: vegetable oil soapstock. Fuel Processing Technology, 86(10), 1087-1096.

Haiduc, A., Brandenberger, M., Suquet, S., Vogel, F., Bernier-Latmani, R., Ludwig, C. 2009. SunCHem: an integrated process for the hydrothermal production of methane from microalgae and CO2 mitigation. Journal of Applied Phycology, 21(5), 529-541.

Halim, R., Danquah, M.K., Webley, P.A. 2012. Extraction of oil from microalgae for biodiesel production: A review. Biotechnology Advances, 30(3), 709-732.

Halim, R., Gladman, B., Danquah, M.K., Webley, P.A. 2011. Oil extraction from microalgae for biodiesel production. Bioresource Technology, 102(1), 178-185.

Heaven, S., Milledge, J., Zhang, Y. 2011. Comments on 'Anaerobic digestion of microalgae as a necessary step to make microalgal biodiesel sustainable'. Biotechnology Advances, 29(1), 164-167.

Henze, M., International Water Association. Task Group on Mathematical Modelling for, D., Operation of Biological Wastewater, T. 2000. Activated sludge models ASM1, ASM2, ASM2d and ASM3. IWA Publ, London.

Ho, S.-H., Chen, W.-M., Chang, J.-S. 2010. Scenedesmus obliquus CNW-N as a potential candidate for CO2 mitigation and biodiesel production. Bioresource Technology, 101(22), 8725-8730.

Hogg, N., Kalyanaraman, B. 1999. Nitric oxide and lipid peroxidation. Biochimica et Biophysica Acta (BBA) - Bioenergetics, 1411(2–3), 378-384.

Holmberg, A. 1982. On the practical identifiability of microbial growth models incorporating Michaelis-Menten type nonlinearities. Mathematical Biosciences, 62(1), 23-43.

Hosseini Tafreshi, A., Shariati, M. 2009. Dunaliella biotechnology: methods and applications. Journal of Applied Microbiology, 107(1), 14-35.

Hu, H., Gao, K. 2006. Response of Growth and Fatty Acid Compositions of Nannochloropsis sp. to Environmental Factors Under Elevated CO2 Concentration. Biotechnology Letters, 28(13), 987-992.

Hu, Q., Sommerfeld, M., Jarvis, E., Ghirardi, M., Posewitz, M., Seibert, M., Darzins, A. 2008. Microalgal triacylglycerols as feedstocks for biofuel production: perspectives and advances. The Plant Journal, 54(4), 621-639.

Jiang, G., Gutierrez, O., Yuan, Z. 2011. The strong biocidal effect of free nitrous acid on anaerobic sewer biofilms. Water Res, 45(12), 3735-43.

Jin, G., Yang, F., Hu, C., Shen, H., Zhao, Z.K. 2012. Enzyme-assisted extraction of lipids directly from the culture of the oleaginous yeast Rhodosporidium toruloides. Bioresource Technology, 111(0), 378-382.

Jupsin, H., Praet, E., Vasel, J. 2003. Dynamic mathematical model of high rate algal ponds (HRAP). Water science and technology: a journal of the International Association on Water Pollution Research, 48(2), 197.

Keymer, P., Lant, P., Pratt, S. 2013a. Modelling microalgal activity as a function of inorganic carbon concentration: accounting for the impact of pH on the bicarbonate system. Journal of Applied Phycology, 1-8.

Keymer, P., Ruffell, I., Pratt, S., Lant, P. 2013b. High pressure thermal hydrolysis as pre-treatment to increase the methane yield during anaerobic digestion of microalgae. Bioresource Technology, 131(0), 128-133.

Keymer, P.C., Pratt, S., Lant, P.A. 2013c. Development of a novel electrochemical system for oxygen control (ESOC) to examine dissolved oxygen inhibition on algal activity. Biotechnology and Bioengineering, 110(9), 2405-2411.

Kim, D.-G., La, H.-J., Ahn, C.-Y., Park, Y.-H., Oh, H.-M. 2011. Harvest of Scenedesmus sp. with bioflocculant and reuse of culture medium for subsequent high-density cultures. Bioresource Technology, 102(3), 3163-3168.

Kim, H.-J., Kang, B.-S., Kim, M.-J., Park, Y.M., Kim, D.-K., Lee, J.-S., Lee, K.-Y. 2004. Transesterification of vegetable oil to biodiesel using heterogeneous base catalyst. Catalysis Today, 93-95, 315-320.

Kim, J., Yoo, G., Lee, H., Lim, J., Kim, K., Kim, C.W., Park, M.S., Yang, J.-W. 2013. Methods of downstream processing for the production of biodiesel from microalgae. Biotechnology Advances, 31(6), 862–876.

Kuentzel, L.E. 1969. Bacteria, Carbon Dioxide, and Algal Blooms. Journal (Water Pollution Control Federation), 41(10), 1737-1747.

Kumar, A., Ergas, S., Yuan, X., Sahu, A., Zhang, Q., Dewulf, J., Malcata, F.X., van Langenhove, H. 2010. Enhanced CO2 fixation and biofuel production via microalgae: recent developments and future directions. Trends in biotechnology, 28(7), 371-380.

Lardon, L., Hélias, A., Sialve, B., Steyer, J.-P., Bernard, O. 2009. Life-Cycle Assessment of Biodiesel Production from Microalgae. Environmental Science & Technology, 43(17), 6475-6481.

Lee, Kim, Kwon, Yoon, Oh. 1998. Effects of harvesting method and growth stage on the flocculation of the green alga Botryococcus braunii. Letters in Applied Microbiology, 27(1), 14-18.

Lee, J.-Y., Yoo, C., Jun, S.-Y., Ahn, C.-Y., Oh, H.-M. 2010. Comparison of several methods for effective lipid extraction from microalgae. Bioresource Technology, 101(1, Supplement), S75-S77.

Li, Y., Horsman, M., Wang, B., Wu, N., Lan, C. 2008. Effects of nitrogen sources on cell growth and lipid accumulation of green alga Neochloris oleoabundans. Applied Microbiology and Biotechnology, 81(4), 629-636.

Ma, Z., Gao, K. 2010. Spiral breakage and photoinhibition of Arthrospira platensis (Cyanophyta) caused by accumulation of reactive oxygen species under solar radiation. Environmental and Experimental Botany, 68(2), 208-213.

Macías-Sánchez, M.D., Mantell, C., Rodríguez, M., Martínez de la Ossa, E., Lubián, L.M., Montero,

O. 2007. Supercritical fluid extraction of carotenoids and chlorophyll a from Synechococcus sp. The Journal of Supercritical Fluids, 39(3), 323-329.

Maeda, K., Owada, M., Kimura, N., Omata, K., Karube, I. 1995. CO2 fixation from the flue gas on coal-fired thermal power plant by microalgae. Energy Conversion and Management, 36(6–9), 717-720.

Mahdy, A., Mendez, L., Ballesteros, M., González-Fernández, C. 2014. Enhanced methane production of Chlorella vulgaris and Chlamydomonas reinhardtii by hydrolytic enzymes addition. Energy Conversion and Management, 85, 551-557.

Mairet, F., Bernard, O., Masci, P., Lacour, T., Sciandra, A. 2011. Modelling neutral lipid production by the microalga Isochrysis aff. galbana under nitrogen limitation. Bioresource Technology, 102(1), 142-149.

Marzano, C.M.A.D.S., Legros, A., Naveau, H.P., Nyns, E.J. 1982. Biomethanation of the Marine Algae Tetraselmis. International Journal of Solar Energy, 1(4), 263-272.

Materials, A.S.f.T. 2009. ASTM D6751-09, Standard Specification for Biodiesel Fuel Blend Stock (B100) for Middle Distillate Fuels, ASTM West Conshohocken, PA.

Medina, M., Neis, U. 2007. Symbiotic algal bacterial wastewater treatment: effect of food to microorganism ratio and hydraulic retention time on the process performance. Water science and technology, 55(11), 165-171.

Mendes-Pinto, M.M., Raposo, M.F.J., Bowen, J., Young, A.J., Morais, R. 2001. Evaluation of different cell disruption processes on encysted cells of Haematococcus pluvialis: effects on astaxanthin recovery and implications for bio-availability. Journal of Applied Phycology, 13(1), 19-24.

Mendes, R.L., Nobre, B.P., Cardoso, M.T., Pereira, A.P., Palavra, A.F. 2003. Supercritical carbon dioxide extraction of compounds with pharmaceutical importance from microalgae. Inorganica Chimica Acta, 356(0), 328-334.

Mesplé, F., Casellas, C., Troussellier, M., Bontoux, J. 1996. Modelling orthophosphate evolution in a high rate algal pond. Ecological Modelling, 89(1-3), 13-21.

Metting, B., Pyne, J.W. 1986. Biologically active compounds from microalgae. Enzyme and Microbial Technology, 8(7), 386-394.

Metting, F.B. 1996. Biodiversity and application of microalgae. Journal of Industrial Microbiology & Biotechnology, 17(5), 477-489.

Metzger, P., Largeau, C. 2005. Botryococcus braunii: a rich source for hydrocarbons and related ether lipids. Applied Microbiology and Biotechnology, 66(5), 486-496.

Milledge, J., Heaven, S. 2013. A review of the harvesting of micro-algae for biofuel production. Reviews in Environmental Science and Bio/Technology, 12(2), 165-178.

Miranda, J.R., Passarinho, P.C., Gouveia, L. 2012. Pre-treatment optimization of Scenedesmus obliquus microalga for bioethanol production. Bioresource Technology, 104(0), 342-348.

Moore, A. 2001. Blooming prospects? EMBO reports, 2(6), 462-464.

Munack, A. 2006. Biodiesel – A comprehensive handbook. Martin Mittelbach, Claudia Remschmidt (Ed.). WILEY-VCH Verlag.

Muñoz, R., Guieysse, B. 2006. Algal-bacterial processes for the treatment of hazardous contaminants: A review. Water Research, 40(15), 2799-2815.

Myers, J., Phillips Jr, J.N., Graham, J.R. 1951. On the mass culture of algae. Plant Physiology, 26(3), 539.

Naveena, B.J., Altaf, M., Bhadrayya, K., Madhavendra, S.S., Reddy, G. 2005. Direct fermentation of starch to l(+) lactic acid in SSF by Lactobacillus amylophilus GV6 using wheat bran as support and substrate: medium optimization using RSM. Process Biochemistry, 40(2), 681-690.

Oswald, W., Gotaas, H., Golueke, C., Kellen, W., Gloyna, E., Hermann, E. 1957. Algae in Waste Treatment [with Discussion]. Sewage and Industrial Wastes, 29(4), 437-457.

Oswald, W.J. 1962. The coming industry of controlled photosynthesis. American Journal of Public Health, 52(2), 235.

Oswald, W.J., Gotaas, H.B. 1957. Photosynthesis in sewage treatment. Trans. Am. Soc. Civ. Eng, 122, 73-105.

Packer, A., Li, Y., Andersen, T., Hu, Q., Kuang, Y., Sommerfeld, M. 2011. Growth and neutral lipid synthesis in green microalgae: A mathematical model. Bioresource Technology, 102(1), 111-117.

Park, J.B.K., Craggs, R.J. 2010. Wastewater treatment and algal production in high rate algal ponds with carbon dioxide addition. Water science and technology, 61(3), 633-639.

Park, J.B.K., Craggs, R.J., Shilton, A.N. 2011. Wastewater treatment high rate algal ponds for biofuel production. Bioresource Technology, 102(1), 35-42.

Passos, F., Hernández-Mariné, M., García, J., Ferrer, I. 2014. Long-term anaerobic digestion of microalgae grown in HRAP for wastewater treatment. Effect of microwave pretreatment. Water Research, 49(0), 351-359.

Passos, F., Solé, M., García, J., Ferrer, I. 2013. Biogas production from microalgae grown in wastewater: Effect of microwave pretreatment. Applied Energy, 108(0), 168-175.

Peng, L., Lan, C.Q., Zhang, Z. 2013. Evolution, detrimental effects, and removal of oxygen in microalga cultures: A review. Environmental Progress & Sustainable Energy, 32(4), 982-988.

Porter, K.G., Feig, Y.S. 1980. The use of DAPI for identifying and counting aquatic microflora. Limnology and Oceanography, 943-948.

Prabakaran, P., Ravindran, A.D. 2011. A comparative study on effective cell disruption methods for lipid extraction from microalgae. Letters in Applied Microbiology, 53(2), 150-154.

Pulz. 2001. Photobioreactors: production systems for phototrophic microorganisms. Applied Microbiology and Biotechnology, 57(3), 287-293.

Pushparaj, B., Pelosi, E., Torzillo, G., Materassi, R. 1993. Microbial biomass recovery using a synthetic cationic polymer. Bioresource Technology, 43(1), 59-62.

Rao, A.R., Dayananda, C., Sarada, R., Shamala, T.R., Ravishankar, G.A. 2007. Effect of salinity on growth of green alga Botryococcus braunii and its constituents. Bioresource Technology, 98(3), 560-564.

Ras, M., Lardon, L., Bruno, S., Bernet, N., Steyer, J.-P. 2011. Experimental study on a coupled process of production and anaerobic digestion of Chlorella vulgaris. Bioresource Technology, 102(1), 200-206.

Raunkjær, K., Hvitved-Jacobsen, T., Nielsen, P.H. 1994. Measurement of pools of protein, carbohydrate and lipid in domestic wastewater. Water Research, 28(2), 251-262.

Rawat, I., Ranjith Kumar, R., Mutanda, T., Bux, F. 2011. Dual role of microalgae: Phycoremediation of domestic wastewater and biomass production for sustainable biofuels production. Applied Energy, 88(10), 3411-3424.

Reichert, P. 1998. Aquasim 2.0--User Manual, Computer Program for the Identification and Simulation of Aquatic Systems.

Reichert, P. 2001. River Water Quality Model no. 1 (RWQM1): case study II. Oxygen and nitrogen conversion processes in the River Glatt (Switzerland). Water science and technology : a journal of the International Association on Water Pollution Research, 43(5), 51-60.

Reichert, P., Borchardt, D. 2001. River Water Quality Model no. 1 (RWQM1): case study. I. Compartmentalisation approach applied to oxygen balances in the River Lahn (Germany). Water science and technology : a journal of the International Association on Water Pollution Research, 43(5), 41-49.

Reichert, P., Borchardt, D., Somlyódy, L., Shanahan, P., Rauch, W., Vanrolleghem, P., Henze, M. 2001a. River Water Quality Model no. 1 (RWQM1): I. Modelling approach. Water science and technology : a journal of the International Association on Water Pollution Research, 43(5), 1-9.

Reichert, P., Borchardt, D., Somlyódy, L., Shanahan, P., Rauch, W., Vanrolleghem, P., Henze, M. 2001b. River Water Quality Model no. 1 (RWQM1): II. Biochemical process equations. Water science and technology : a journal of the International Association on Water Pollution Research, 43(5), 11-30.

Reichert, P., Borchardt, D., Somlyódy, L., Shanahan, P., Rauch, W., Vanrolleghem, P., Henze, M. 2001c. River Water Quality Model no. 1 (RWQM1): III. Biochemical submodel selection. Water science and technology : a journal of the International Association on Water Pollution Research, 43(5), 31-40.

Reichert, P., Vanrolleghem, P. 2001. Identifiability and uncertainty analysis of the river water quality model no. 1 (RWQM1). Water science and technology : a journal of the International Association on Water Pollution Research, 43(7), 329-338.

Richmond, A. 2004. Handbook of Microalgal Culture: Biotechnology and Applied Phycology. Wiley-Blackwell.

Riquelme, C.E., Fukami, K., Ishida, Y. 1987. Annual Fluctuations of phytoplankton and bacterial communities in Maizuru bay and their interrelationship. Bulletin of Japanese Society of Microbial Ecology, 2(1), 29-37.

Rodolfi, L., Chini Zittelli, G., Bassi, N., Padovani, G., Biondi, N., Bonini, G., Tredici, M.R. 2009.

Microalgae for oil: Strain selection, induction of lipid synthesis and outdoor mass cultivation in a low-cost photobioreactor. Biotechnology and Bioengineering, 102(1), 100-112.

Rösch, C., Skarka, J., Wegerer, N. 2012. Materials flow modeling of nutrient recycling in biodiesel production from microalgae. Bioresource Technology, 107(0), 191-199.

Rotatore, C., Colman, B. 1991a. The active uptake of carbon dioxide by the unicellular green algae Chlorella saccharophila and C. ellipsoidea. Plant, Cell & Environment, 14(4), 371-375.

Rotatore, C., Colman, B. 1991b. The localization of active inorganic carbon transport at the plasma membrane in Chlorella ellipsoidea. Canadian Journal of Botany, 69(5), 1025-1031.

Ryu, H.J., Oh, K.K., Kim, Y.S. 2009. Optimization of the influential factors for the improvement of CO2 utilization efficiency and CO2 mass transfer rate. Journal of Industrial and Engineering Chemistry, 15(4), 471-475.

Salim, S., Bosma, R., Vermuë, M., Wijffels, R. 2010. Harvesting of microalgae by bio-flocculation. Journal of Applied Phycology, 1-7.

Sánchez Mirón, A., Contreras Gómez, A., García Camacho, F., Molina Grima, E., Chisti, Y. 1999. Comparative evaluation of compact photobioreactors for large-scale monoculture of microalgae. Journal of Biotechnology, 70(1–3), 249-270.

Sander, K., Murthy, G.S. 2010. Life cycle analysis of algae biodiesel. The International Journal of Life Cycle Assessment, 1-11.

Sander, R. 1999. Compilation of Henry's law constants for inorganic and organic species of potential importance in environmental chemistry, Max-Planck Institute of Chemistry, Air Chemistry Department.

Santabarbara, S., Cazzalini, I., Rivadossi, A., Garlaschi, F.M., Zucchelli, G., Jennings, R.C. 2002. Photoinhibition in vivo and in vitro Involves Weakly Coupled Chlorophyll–Protein Complexes†¶. Photochemistry and Photobiology, 75(6), 613-618.

Sarada, R., Vidhyavathi, R., Usha, D., Ravishankar, G.A. 2006. An Efficient Method for Extraction of Astaxanthin from Green Alga Haematococcus pluvialis. Journal of Agricultural and Food Chemistry, 54(20), 7585-7588.

Schenk, P., Thomas-Hall, S., Stephens, E., Marx, U., Mussgnug, J., Posten, C., Kruse, O., Hankamer, B. 2008. Second Generation Biofuels: High-Efficiency Microalgae for Biodiesel Production. BioEnergy Research, 1(1), 20-43.

Scott, S.A., Davey, M.P., Dennis, J.S., Horst, I., Howe, C.J., Lea-Smith, D.J., Smith, A.G. 2010. Biodiesel from algae: challenges and prospects. Current Opinion in Biotechnology, 21(3), 277-286.

Shieh, C.J., Liao, H.F., Lee, C.C. 2003. Optimization of lipase-catalyzed biodiesel by response surface methodology. Bioresource Technology, 88(2), 103-106.

Sialve, B., Bernet, N., Bernard, O. 2009. Anaerobic digestion of microalgae as a necessary step to make microalgal biodiesel sustainable. Biotechnology Advances, 27(4), 409-416.

Singh, A., Nigam, P.S., Murphy, J.D. 2011. Mechanism and challenges in commercialisation of algal biofuels. Bioresource Technology, 102(1), 26-34.

Singh, A., Olsen, S.I. 2011. A critical review of biochemical conversion, sustainability and life cycle assessment of algal biofuels. Applied Energy, 88(10), 3548-3555.

Singh, J., Gu, S. 2010. Commercialization potential of microalgae for biofuels production. Renewable and Sustainable Energy Reviews, 14(9), 2596-2610.

Smith, P.K., Krohn, R.I., Hermanson, G.T., Mallia, A.K., Gartner, F.H., Provenzano, M.D., Fujimoto, E.K., Goeke, N.M., Olson, B.J., Klenk, D.C. 1985. Measurement of protein using bicinchoninic acid. Analytical Biochemistry, 150(1), 76-85.

Spilling, K., Seppälä, J., Tamminen, T. 2010. Inducing autoflocculation in the diatom Phaeodactylum tricornutum through CO2 regulation. Journal of Applied Phycology, 1-8.

Spolaore, P., Joannis-Cassan, C., Duran, E., Isambert, A. 2006. Commercial applications of microalgae. Journal of Bioscience and Bioengineering, 101(2), 87-96.

Stephenson, A.L., Dennis, J.S., Howe, C.J., Scott, S.A., Smith, A.G. 2010. Influence of nitrogen-limitation regime on the production by Chlorella vulgaris of lipids for biodiesel feedstocks. Biofuels, 1(1), 47-58.

Sukenik, A., Shelef, G. 1984. Algal autoflocculation—verification and proposed mechanism. Biotechnology and Bioengineering, 26(2), 142-147.

Suminto, Hirayama, K. 1997. Application of a growth-promoting bacteria for stable mass culture of three marine microalgae. Hydrobiologia, 358(1), 223-230.

Torres-Martínez, D., Melgarejo-Torres, R., Gutiérrez-Rojas, M., Aguilera-Vázquez, L., Micheletti, M., Lye, G.J., Huerta-Ochoa, S. 2009. Hydrodynamic and oxygen mass transfer studies in a three-phase (air–water–ionic liquid) stirred tank bioreactor. Biochemical Engineering Journal, 45(3), 209-217.

Torzillo, G., Pushparaj, B., Bocci, F., Balloni, W., Materassi, R., Florenzano, G. 1986. Production of Spirulina biomass in closed photobioreactors. Biomass, 11(1), 61-74.

Tugtas, A.E., Pavlostathis, S.G. 2007. Inhibitory effects of nitrogen oxides on a mixed methanogenic culture. Biotechnology and Bioengineering, 96(3), 444-455.

Uggetti, E., Sialve, B., Trably, E., Steyer, J.-P. 2014. Integrating microalgae production with anaerobic digestion: a biorefinery approach. Biofuels, Bioproducts and Biorefining, 8(4), 516-529.

Ugwu, C.U., Aoyagi, H., Uchiyama, H. 2007. Influence of irradiance, dissolved oxygen concentration, and temperature on the growth of Chlorella sorokiniana. Photosynthetica, 45(2), 309-311.

Vadivelu, V.M., Yuan, Z., Fux, C., Keller, J. 2006. Stoichiometric and kinetic characterisation of Nitrobacter in mixed culture by decoupling the growth and energy generation processes. Biotechnology and Bioengineering, 94(6), 1176-1188.

Vaishampayan, A., Sinha, R., Hader, D., Dey, T., Gupta, A., Bhan, U., Rao, A. 2001. Cyanobacterial biofertilizers in rice agriculture. The Botanical Review, 67(4), 453-516.

van Beilen, J.B. 2010. Why microalgal biofuels won't save the internal combustion machine. Biofuels, Bioproducts and Biorefining, 4(1), 41-52.

Van Den Hende, S., Vervaeren, H., Desmet, S., Boon, N. 2011a. Bioflocculation of microalgae and

bacteria combined with flue gas to improve sewage treatment. New Biotechnology, In Press, Corrected Proof.

Van Den Hende, S., Vervaeren, H., Saveyn, H., Maes, G., Boon, N. 2011b. Microalgal bacterial floc properties are improved by a balanced inorganic/organic carbon ratio. Biotechnology and Bioengineering, 108(3), 549-558.

Venkata Mohan, S., Lalit Babu, V., Sarma, P.N. 2008. Effect of various pretreatment methods on anaerobic mixed microflora to enhance biohydrogen production utilizing dairy wastewater as substrate. Bioresource Technology, 99(1), 59-67.

Wang, L., Weller, C.L. 2006. Recent advances in extraction of nutraceuticals from plants. Trends in Food Science & Technology, 17(6), 300-312.

Wang, Q., Ye, L., Jiang, G., Jensen, P.D., Batstone, D.J., Yuan, Z. 2013. Free Nitrous Acid (FNA)-Based Pretreatment Enhances Methane Production from Waste Activated Sludge. Environmental Science & Technology, 47(20), 11897-11904.

Wang, Y., Duanmu, D., Spalding, M.H. 2011. Carbon dioxide concentrating mechanism in Chlamydomonas reinhardtii: inorganic carbon transport and CO2 recapture. Photosynthesis research, 109(1-3), 115-122.

Warabi, Y., Kusdiana, D., Saka, S. 2004. Reactivity of triglycerides and fatty acids of rapeseed oil in supercritical alcohols. Bioresource Technology, 91(3), 283-287.

Weissman, J.C., Goebel, R.P., Benemann, J.R. 1988. Photobioreactor design: Mixing, carbon utilization, and oxygen accumulation. Biotechnology and Bioengineering, 31(4), 336-344.

Wijffels, R.H., Barbosa, M.J. 2010. An Outlook on Microalgal Biofuels. Science, 329(5993), 796-799.

Wolf, G., Picioreanu, C., van Loosdrecht, M.C.M. 2007. Kinetic modeling of phototrophic biofilms: The PHOBIA model. Biotechnology and Bioengineering, 97(5), 1064-1079.

Xia, J., Wan, M., Wang, R., Liu, P., Li, L., Huang, B., Qiu, G. 2009. Current status and progress of Microalgal Biodiesel. China Biotechnology, 29(7), 118-126.

Xu, H., Miao, X., Wu, Q. 2006. High quality biodiesel production from a microalga Chlorella protothecoides by heterotrophic growth in fermenters. Journal of Biotechnology, 126(4), 499-507.

Yang, A. 2011. Modeling and Evaluation of CO2 Supply and Utilization in Algal Ponds. Industrial & Engineering Chemistry Research, 50(19), 11181-11192.

Yang, J., Xu, M., Zhang, X., Hu, Q., Sommerfeld, M., Chen, Y. 2011. Life-cycle analysis on biodiesel production from microalgae: Water footprint and nutrients balance. Bioresource Technology, 102(1), 159-165.

Yeesang, C., Cheirsilp, B. 2011. Effect of nitrogen, salt, and iron content in the growth medium and light intensity on lipid production by microalgae isolated from freshwater sources in Thailand. Bioresource Technology, 102(3), 3034-3040.

Yen, H.-W., Hu, I.C., Chen, C.-Y., Ho, S.-H., Lee, D.-J., Chang, J.-S. 2013. Microalgae-based biorefinery – From biofuels to natural products. Bioresource Technology, 135, 166-174.

Yoo, C., Jun, S.-Y., Lee, J.-Y., Ahn, C.-Y., Oh, H.-M. 2010. Selection of microalgae for lipid production under high levels carbon dioxide. Bioresource Technology, 101(1, Supplement 1), S71-S74.

Young, G., Nippen, F., Titterbrandt, S., Cooney, M.J. 2011. Direct transesterification of biomass using an ionic liquid co-solvent system. Biofuels, 2(3), 261-266.

Yue, L., Chen, W. 2005. Isolation and determination of cultural characteristics of a new highly CO2 tolerant fresh water microalgae. Energy Conversion and Management, 46(11–12), 1868-1876.

Yun, Y.-S., Park, J., Yang, J.-W. 1996. Enhancement of CO2 tolerance of Chlorella vulgaris by gradual increase of CO2 concentration. Biotechnology Techniques, 10(9), 713-716.

Zhao, B., Su, Y. 2014. Process effect of microalgal-carbon dioxide fixation and biomass production: A review. Renewable and Sustainable Energy Reviews, 31(0), 121-132.

Zhao, G., Du, J., Jia, Y., Lv, Y., Han, G., Tian, X. 2012. The importance of bacteria in promoting algal growth in eutrophic lakes with limited available phosphorus. Ecological Engineering, 42(0), 107-111.

Zheng, H., Yin, J., Gao, Z., Huang, H., Ji, X., Dou, C. 2011. Disruption of Chlorella vulgaris cells for the release of biodiesel-producing lipids: a comparison of grinding, ultrasonication, bead milling, enzymatic lysis, and microwaves. Applied Biochemistry and Biotechnology, 164(7), 1215-1224.

Appendix A

The contribution of bacteria to algal growth by carbon cycling

ARTICLE

BIOTECHNOLOGY AND BIOENGINEERING

The Contribution of Bacteria to Algal Growth by Carbon Cycling

Xue Bai, Paul Lant, Steven Pratt

School of Chemical Engineering, The University of Queensland, St Lucia 4072, Brisbane, Queensland; telephone: +61-7-336-54943; fax: +61-7-3365 4199; e-mail: s.pratt@uq.edu.au

ABSTRACT: Algal mass production in open systems is often limited by the availability of inorganic carbon substrate. In this paper, we evaluate how bacterial driven carbon cycling mitigates carbon limitation in open algal culture systems. The contribution of bacteria to carbon cycling was determined by quantifying algae growth with and without supplementation of bacteria. It was found that adding heterotrophic bacteria to an open algal culture dramatically enhanced algae productivity. Increases in algal productivity due to supplementation of bacteria of 4.8 and 3.4 times were observed in two batch tests operating at two different pH values over 7 days. A kinetic model is proposed which describes carbon limited algal growth, and how the limitation could be overcome by bacterial activity to re-mineralize photosynthetic end products.

Biotechnol. Bioeng. 2014;9999: 1–9.

KEYWORDS: algae; algal cultivation; bacteria; carbon cycling; modeling

Introduction

Open mass algal culture systems are a low cost option for mass algal cultivation. Such systems have been used for the production of biofuels and chemicals, food and animal feeds, and for bioremediation (Brennan and Owende, 2010; Chisti, 2007; Gallagher, 2011; Ho et al., 2010; Muñoz and Guieysse, 2006; Richmond, 1992). This paper addresses a major challenge to algal productivity in these systems, namely ensuring sufficient availability of inorganic carbon substrate. The focus of the work is the quantification of the contribution of bacteria to algal growth by carbon cycling. In short, this work is concerned with elevating net photosynthetic yield by re-mineralizing photosynthetic end products.

Correspondence to: Steven Pratt
Received 1 May 2014; Revision received 14 September 2014; Accepted 26 September 2014
Accepted manuscript online xx Month 2014;
Article first published online in Wiley Online Library (wileyonlinelibrary.com).
DOI 10.1002/bit.25475

The mass transfer of inorganic carbon (carbon dioxide) into open systems is shown as Equation (1):

$$r = K_{LaCO_2}\left(S^*_{CO_2} - S_{CO_2}\right). \quad (1)$$

where K_{LaCO_2} (d^{-1}) is the mass transfer efficiency, $S^*_{CO_2}$ (mg C L^{-1}) is the saturated dissolved CO_2 concentration, and S_{CO_2} (mg C L^{-1}) is the dissolved CO_2 concentration.

Systems that rely on conventional carbon supply strategies (e.g., air bubbling, paddling, etc.) are carbon limited due to (i) low transfer efficiency (low K_{LaCO_2}), which is a function of the aeration infrastructure and reactor configurations as well as the high surface tension of water, and (ii) low driving force due to low $S^*_{CO_2}$, which is a consequence of the relatively low CO_2 content of the atmosphere (0.038% v v^{-1}) (Sander, 1999).

The main strategy to boost the driving force is to use CO_2 enriched gases (such as flue gas). This has been demonstrated at both laboratory scale and pilot scale, where CO_2 addition (up to a point) has led to higher algal photosynthetic efficiencies and productivities compared to controls (Yang, 2011; Zhao and Su, 2014). Azov and Goldman (1982) found that algal production in a pilot-scale high rate algal pond (HRAP) with CO_2 addition was more than double that achieved in one without CO_2 addition.

It is well documented that numerous interactions occur in algal–bacterial systems (Cole, 1982). It is generally thought that the algae and bacteria maintain symbiotic relations. Previous work has shown that the presence of bacteria may aid algal productivity (Andersen, 2005; Fukami et al., 1997; Riquelme et al., 1987; Suminto and Hirayama 1997). The stimulative effect of bacteria on algal growth has been described as being associated with bacteria mineralizing carbon (Ask et al., 2009; Muñoz and Guieysse, 2006), as well as cycling nutrients (Bloesch et al., 1977; Zhao et al., 2012) and generating vitamins (Grant et al., 2014) and other growth promoting regulators. Considering bacteria mineralizing carbon, this carbon cycling process has potential to help overcome carbon limitation in open algal culture systems. Carbon cycling refers to the oxidation of the products of photosynthesis to CO_2, which is then available to be re-processed into organic compounds by further photosynthesis. In open algal culture systems, oxidation is driven by heterotrophic bacteria.

Mineralization of organic carbon underpins the design of some algae-based wastewater treatment systems. Some mathematical

Abstract

Algal mass production in open systems is often limited by the availability of inorganic carbon substrate. In this paper, we evaluate how bacterial driven carbon cycling mitigates carbon limitation in open algal culture systems. The contribution of bacteria to carbon cycling was determined by quantifying algae growth with and without supplementation of bacteria. It was found that adding heterotrophic bacteria to an open algal culture dramatically enhanced algae productivity. Increases in algal productivity due to supplementation of bacteria of 4.8 and 3.4 times were observed in two batch tests operating at two different pH values over 7 days. A kinetic model is proposed which describes carbon limited algal growth, and how the limitation is overcome by bacterial activity to re-mineralise photosynthetic end products.

Keywords: Algae, Bacteria, Carbon cycling, Modelling, Algal cultivation

1. Introduction

Open algal culture systems are a low cost option for mass algal cultivation. Such systems have been used for the production of biofuels and chemicals, food and animal feeds, and for bioremediation (Brennan and Owende, 2010; Chisti, 2007; Gallagher, 2011; Ho et al., 2010; Muñoz and Guieysse, 2006; Richmond, 1992). This paper addresses a major challenge to algal productivity in these systems, namely ensuring sufficient availability of inorganic carbon substrate. The focus of the work is the quantification of the contribution of bacteria to algal growth by carbon cycling. In short, this work is concerned with elevating net photosynthetic yield by re-mineralising photosynthetic end products.

The mass transfer of inorganic carbon (carbon dioxide) into open systems is shown as Eq. 1:

$$r = K_{LaCO2}(S^*_{CO2} - S_{CO2}) \qquad \text{Eq. 1}$$

where K_{LaCO2} (d^{-1}) is the mass transfer efficiency, S^*_{CO2} (mg C L^{-1}) is the saturated dissolved CO_2 concentration, and S_{CO2} (mg C L^{-1}) is the dissolved CO_2 concentration.

Systems that rely on conventional carbon supply strategies (e.g. air bubbling, paddling etc.) are carbon limited due to i) low transfer efficiency (low K_{LaCO2}), which is a function of the aeration infrastructure and reactor configurations as well as the high surface tension of water, and ii) low driving force due to low S^*_{CO2}, which is a consequence of the relatively low CO_2 content of the atmosphere (0.038% v v^{-1}) (Sander, 1999).

The main strategy to boost the driving force is to use CO_2 enriched gases (such as flue gas). This has

been demonstrated at both laboratory scale and pilot scale, where CO_2 addition (up to a point) has led to higher algal photosynthetic efficiencies and productivities compared to controls (Yang, 2011; Zhao and Su, 2014). Azov and Goldman (1982) found that algal production in a pilot-scale high rate algal pond (HRAP) with CO_2 addition was more than double that achieved in one without CO_2 addition.

It is well documented that numerous interactions occur in algal-bacterial systems (Cole 1982). It is generally thought that the algae and bacteria maintain symbiotic relations. Previous work has shown that the presence of bacteria may aid algal productivity (Andersen 2005; Fukami et al. 1997; Riquelme et al. 1987; Suminto and Hirayama 1997). The stimulative effect of bacteria on algal growth has been described as being associated with bacteria mineralising carbon (Ask et al., 2009; Muñoz and Guieysse, 2006), as well as cycling nutrients (Bloesch et al., 1977; Zhao et al., 2012) and generating vitamins (Grant et al., 2014) and other growth promoting regulators. Considering bacteria mineralising carbon, this carbon cycling process has potential to help overcome carbon limitation in open algal culture systems. Carbon cycling refers to the oxidation of the products of photosynthesis to CO_2, which is then available to be re-processed into organic compounds by further photosynthesis. In open algal culture systems, oxidation is driven by heterotrophic bacteria.

Mineralisation of organic carbon underpins the design of some algae-based wastewater treatment systems. Some mathematical models of these systems, including high rate algal-bacterial ponds, consider links between algae and bacteria (Buhr and Miller, 1983; Jupsin et al., 2003; Yang, 2011) with Yang (2011) even focusing on CO_2 supply and utilization in these systems. Yang showed that bacterial driven carbon oxidation, albeit oxidation of supplemented organic carbon, can play an important role in supporting algal activity. However, neither Yang (2011) nor the other authors, have quantified the contribution of bacterial carbon cycling (that is oxidation of products of photosynthesis) to algal productivity.

In this paper, the contribution of bacteria to open algal cultivation is evaluated. Emphasis is on quantification of the effect of bacterial carbon cycling on algal productivity. Two batches of experiments were performed to quantify algal biomass productivity with and without supplementation of heterotrophic bacteria. A model is proposed which describes inorganic carbon limitation for algal growth and how the limitation is overcome by bacterial activity to re-mineralise photosynthetic end products.

2. Materials and Methods

2.1. Organisms and reactor operation

Chlorella sp. was pre-cultured in a 3 L photobioreactor for three months and acclimatized by feeding f/2 media (Varicon Aqua, Worcestershire, UK). Heterotrophic bacteria inoculum was obtained from a sequencing batch reactor treating synthetic domestic wastewater, maintained at The University of Queensland, Australia. The ratio of supplemented bacterial biomass to algal biomass was 1:10 (dry mass basis). Before inoculation, both algae and bacteria inoculum were centrifuged and washed 3 times with Milli-Q water (Milli-Q® Direct Water Purification System, Merck Millipore Pty. Ltd., Germany) to remove nutrients and vitamins from the inoculum. f/2 media was added to the reactors to supply sufficient nutrients for algal growth. Nutrients for algal growth were monitored by off-line analysis (described in section 2.4) to ensure that they were in excess.

Two batch tests, carried out at two different pH values, were conducted to examine the effect of the bacteria supplementation (Table II). Batch 1 was operated at pH 8.5 in triplicate for 7 days. Six reactors were run in parallel with two different initial bacteria to algae ratios (Exp.1 and Exp.2). Batch 2 was operated at pH 7.5 in duplicate and cultivation time was extended to 9 days. Four reactors were run with two different initial bacteria to algae ratios (Exp.3 and Exp.4). The growth media was buffered by 20 mM HEPES (Sigma-Aldrich Pty. Ltd) at 8.5±0.2 for Batch 1 and 7.5±0.2 for Batch 2. HEPES is an organic zwitterionic buffer commonly used in algae growth studies. HEPES is not readily consumed by bacteria (this was verified in a separate study [data not shown]). The pH was monitored with a portable pH probe. All of the bench-scale growth reactors were 330 mL glass reactors, well mixed by magnetic stirrers at a constant speed of 350 rpm. Light was generated by a 36 W fluorescent lamp (Osram, 18 W/840) from the top of the reactors. Lighting was operated on a 12 h light, 12 h dark period, and the light intensity was measured to be 180 μmol photons m^{-2} s^{-1} at the incident sides of the reactors by a light meter (840020C, Sper Scientific Ltd., USA). The experiment was operated in a temperature-controlled room with constant ambient temperature at 23 ± 0.2 ˚C.

Table II: Experimental conditions of two batch experiments, including the initial conditions of state variables in the model. X_{alg} and X_{bac} were quantified by TSS measurement. S_{CO2}, S_{HCO3} and S_{CO3} were calculated by TIC values and measured pH.

Initial conditions	**Batch 1 (pH 8.5)**		**Batch 2 (pH 7.5)**	
	Exp.1	**Exp.2**	**Exp.3**	**Exp.4**
X_{alg} **(mg L^{-1})**	90.0	90.0	86.6	86.6
X_{bac} **(mg L^{-1})**	1.00	9.10	1.00	8.60
Bacterial density (%)	1.1	9.2	1.1	9.0
S_{CO2} **(mg C L^{-1})**	0.13	0.13	1.24	1.24
S_{HCO3} **(mg C L^{-1})**	18.03	18.03	17.16	17.16
S_{CO3} **(mg C L^{-1})**	0.27	0.27	0.03	0.03

2.2. Data collection

Samples were collected every 24 hours from all reactors. Optical density at wavelength 680 nm (OD_{680}) was measured by a spectrophotometer (Varian Cary® 50, Varian, Inc., Australia) in a cuvette with a 1 cm light path. Dry algal biomass was determined gravimetrically by filtering 20 mL of culture through a GF/C filter (Whatman) which was rinsed with deionized water, dried in a 105 ˚C oven for 12 h, cooled in a silica gel desiccator and weighted to determine the algal dry weight. A linear relationship between OD_{680} and dry algal biomass was determined prior to the test. Separate study was conducted to verify that there was negligible interference from bacterial density to the OD measurement of algal biomass within the time frame of this study (data not shown).

OD_{680} and algal biomass dry weight from five different culture densities was measured and correlated with a linear correlation (Eq. 2), where m and n in the equation vary between different algal species. Preliminary tests established the linear correlation with m = 342.91 and n = -10.19 respectively at R^2 = 0.98. Bacteria did not affect the optical density measurement under 680nm wavelength (tested in a separate study).

$$Dry\ weight\ of\ algae\ (mg\ DW\ L^{-1}) = m \times OD_{680} + n \qquad \text{Eq. 2}$$

2.3. Microscope observation

Light microscope observation on algal and bacterial cells was performed during cultivation. 1 mL of sample from every reactor was diluted 2 times before staining by adding 1 mL of Milli-Q water. 1 mL of diluted sample was stained with DAPI nuclear fluorescence microprobe (Invitrogen, Ltd., UK), according to a procedure modified from Porter and Feig (1980). The DAPI stain was supplied as a 1 mg mL^{-1} stock solution. 20 µL of the stock solution was added to 1 mL cell suspension giving a final dye concentration of 20 µg mL^{-1}, and the mixture was incubated for 7 minutes at room temperature in the dark, and then filtered. Stained samples were filtered through 0.2 µm black Nuclepore Polycarbonate filters (Advantec®, MFS, Inc., Japan) mounted onto Polypropylene In-Line filter holders (Advantec®, MFS, Inc., Japan). Filters were taken from the filtration unit and then mounted onto glass slides. The algal and bacterial cells were then examined and observed in random fields under a fluorescence microscope (BX61, Olympus, Tokyo, Japan) equipped with a double band pass filter set at 473-498 and 548-573 nm for excitation, and at 515-535 and 590-620 nm for emission. The DAPI stain applied has a high affinity to nucleic acid in the bacterial cells and can emit bright blue fluorescence light when excited with a 450-490nm source (Porter and Feig, 1980), and autofluorescence of chlorophyll was observed simultaneously as red colour, which allows the discrimination of bacterial and algal cells. Images were obtained and post-adjusted with DPC controller 1.2.1 (Olympus optical co. Ltd., Japan) and iTEM 5.0 (Olympus Soft Imagining Solutions, Olympus optical co. Ltd., Japan) respectively.

2.4. Analytical methods

NH_4^+-N, NO_3^--N and PO_4^{3-}-P were measured with a Lachat QuikChem 8000 Flow Injection Analyser (Lachat Instrument, Milwau-kee, USA). Total inorganic carbon (TIC) was measured on a Total Organic Carbon Analyser (Analytik Jena multi N/C 2100S) by injecting a filtered 250 µL sample into a 2.6 M phosphoric acid solution from which the formed CO_2 was stripped and detected with a near infrared detector in a stream of O_2 (160 mL min^{-1}). Biomass

elemental composition (C, H, O, N and ash) of algal inoculum was quantified by a TruSpec LECO CHN analyser (St Joseph, MI, USA). Samples were centrifuged for 10 mins at 3270 g (Beckman Coulter, Allegra™ X-12) and the resulting pellets were dried in a 105 ˚C oven over night. The dry biomass powder was combusted at 925 ˚C in the oven of the elemental analyser. Ash content was determined by burning the dried algae samples in an oven at 550 ˚C, so that all organic material was oxidized and the ash residue remained. From these determinations the O content and subsequently the dry biomass molar mass was calculated.

3. Modelling

The model describing algal growth is based on Monod kinetics by inorganic carbon limitation. Since algal growth in this study was limited by the availability of dissolved CO_2, other limiting effects were eliminated by ensuring sufficient nutrient availability, light, and enough trace elements and vitamins. Figure 1 illustrates the conceptual model developed for this system. The model consists of 5 state variables (S_{CO2}, S_{HCO3}, S_{CO3}, X_{alg}, X_{bac}), 2 biological algal processes (growth and death of algae), 1 bacterial process (net growth of bacteria), as well as 3 chemical-physical processes. Process 1, the growth of algae (X_{alg}), is described as photosynthesis by algae and is limited by the availability of CO_2 (S_{CO2}) and light intensity. To apply the model to other algae species with affinity for HCO_3^-, the kinetic expressions for algal growth may need to be adjusted by considering both CO_2 and HCO_3^- as carbon substrates. Death of algae (Process 2) is the process of conversion of algal biomass (X_{alg}) to slowly degradable and other organic matter by decay and lysis (Van Loosdrecht and Henze, 1999). Net bacterial growth is presented as Process 3, which describes the aerobic process of bacteria oxidising dissolved organic carbon to CO_2. Processes 4-6 represent gas-liquid mass transfer of CO_2 and chemical equilibria of the bicarbonate system. The entire model, with process and rate expressions, is presented in the Petersen matrix in Table I.

Table I: Petersen's matrix for the applied model in this study

Component *i* →		**1**	**2**	**3**	**4**	**5**	**Rates**
Process *j* ↓		**S_{CO2}**	**S_{HCO3}**	**S_{CO3}**	**X_{alg}**	**X_{bac}**	
1	Growth of algae with CO_2	$-a_{C,alg}$ (1)			1		$\mu_{max,alg}\frac{S_{CO2}}{S_{CO2}+K_{CO2,alg}}\frac{I_{ave}}{I_{opt}}e^{\left(1-\frac{I_{ave}}{I_{opt}}\right)}X_{alg}$
2	Death of algae	$a_{C,alg}$-0.21 (2)			-1		$b_{alg}X_{alg}$
3	Net growth of bacteria (3)	0.43				1	$\mu_{bac}X_{bac}$
4	Equilibrium $CO_2 \rightarrow HCO_3^-$	-1	1				$k1 \times \left(S_{CO2} - S_H \times \frac{S_{HCO3}}{K_{eq1}}\right)$ (4)
5	Equilibrium $HCO_3^- \rightarrow CO_3^{2-}$		-1	1			$k2 \times \left(S_{HCO3} - S_H \times \frac{S_{CO3}}{K_{eq2}}\right)$ (5)
6	Mass transfer of CO_2	1					$K_{LaCO2}(S^*_{CO2} - S_{CO2})$ (6)

(1) Mass fraction of algae was measured in this study by elemental analysis.

(2) Stoichiometric parameter of death of algae was determined by the carbon mass fraction of algal biomass and the conversion of algal biomass to inert particulate organic material or biodegradable particulate organic material (Reichert et al. 2001).

(3) This process was only activated in Exp.2 and Exp.4. Net growth rate of bacteria μ_{bac} lumped the growth of bacteria and the death of bacteria.

(4) $K_{eq1} = S_{HCO3} \cdot \frac{S_H}{S_{CO2}}$, $S_H = 10^{-pH}$.

(5) $K_{eq2} = S_{CO3} \cdot \frac{S_H}{S_{HCO3}}$.

(6) $KLa_{CO2} = KLa_{O2}\sqrt{\frac{D_{CO2}}{D_{O2}}}$. KLa_{O2}, the mass transfer coefficient of O_2, was obtained by the dynamic method of measuring DO with an optical DO probe (AquaPlus, Aquaread Ltd., UK) (Torres-Martínez et al. 2009). D_{CO2} and D_{O2} as 1.97×10^{-9} and 2.1×10^{-9} d^{-1} are the diffusion coefficient of CO_2 and O_2 in water respectively at 25 ºC (Wolf et al. 2007). S^*_{CO2} (mg C L^{-1}) is the saturated CO_2 concentration in the solution. It is calculated from the atmospheric concentration of CO_2 and the Henry constant (Sander 1999).

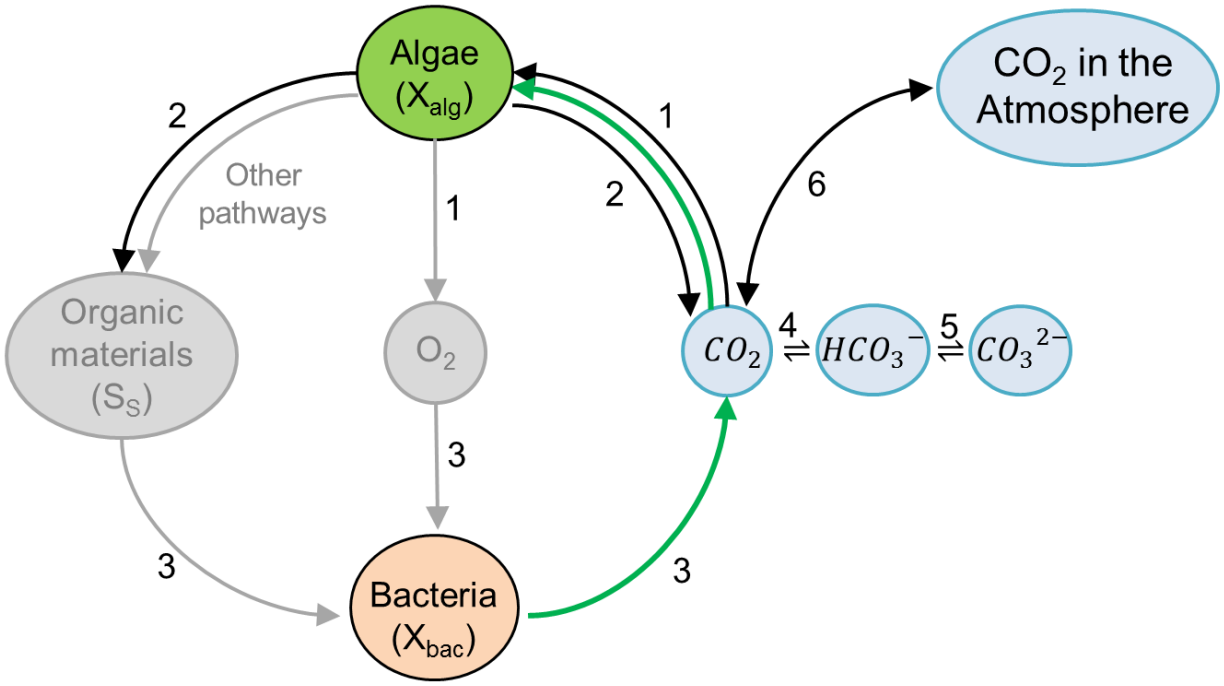


Figure 1. Conceptual model of carbon recycle by bacteria in open algal culture systems.

Light penetration was modelled by the Lambert-Beer law as described in Eq. 3 (Huisman, 1999).

$$\mu = \mu_{max} \frac{I_{ave}}{I_{opt}} e^{\left(1-\frac{I_{ave}}{I_{opt}}\right)} \quad \text{Eq. 3}$$

where I_{opt} (µmol photons m^{-2} s^{-1}) is the optimum light intensity, I_{ave} (µmol photons m^{-2} s^{-1}) is the average light intensity penetrating into the reactor.

In order to find the average light intensity, integration over the path length (z) inside the reactor is required. At a depth of z (m), the irradiance is related to the incident irradiance I_0 (µmol photons m^{-2} s^{-1}) by the function $I(z) = I_0 exp(-aX_{alg}z)$, where X_{alg} is the algae density and a (m^2 mg^{-1}) is the absorption coefficient of the algae. Therefore, the average light intensity can be expressed as Eq. 4 (Barbosa et al., 2003).

$$I_{ave} = I_0 \cdot \frac{1}{z}(1 - e^{-a \cdot X_{alg} \cdot z}) \cdot \frac{1}{X_{alg} a} \quad \text{Eq. 4}$$

Table III defines all of the stoichiometric and kinetic parameters used in the model. Most parameters are taken from literature (Reichert et al., 2001), but two key parameters, $\mu_{max,alg}$ and μ_{bac}, were estimated in this study using the experimental data using a least squares parameter estimation, and two others, $a_{C,alg}$ and K_{LaO2}, were calibrated for this work. The Petersen matrix format enables the mass balance for each state variable to be easily written. As shown in Eq. 5, the mass balance of

component i is obtained by summing the products of the stoichiometric coefficients ϑ_{ij} and the process rate expressions r_j. For example, the accumulation algal biomass (X_{alg}) is obtained by considering the growth of algae and the death of algae (Eq. 6).

$$\frac{di(t)}{dt} = \sum_j \vartheta_{ij} \cdot r_j \qquad \text{Eq. 5}$$

$$\frac{dX_{alg}(t)}{dt} = \mu_{max,alg} \frac{S_{CO2}}{S_{CO2}+K_{CO2,alg}} \frac{I_{ave}}{I_{opt}} e^{(1-\frac{I_{ave}}{I_{opt}})} X_{alg}(t) - b_{alg} X_{alg}(t) \qquad \text{Eq. 6}$$

The parameter estimations and model simulations were performed using the software package AQUASIM (Reichert, 1998). MATLAB (MathWorks, Inc., US) was applied to generate 3D-plots and contour curves. The 3D-plots show simulation results of algal biomass productivity R_{alg} (mg DW L^{-1} d^{-1}) (defined by Eq. 7) with for a range of pH values (6-11) along a 10-day cultivation.

$$R_{alg} = \mu_{max,alg} \frac{S_{CO2}}{S_{CO2}+K_{CO2,alg}} X_{alg} \qquad \text{Eq. 7}$$

Table III: Stoichiometric and kinetic parameters used in the model and the source of value.

Parameter	Definition	Value	Units	Source
Estimated parameters in this study				
$\mu_{max,alg}$	Maximum specific growth rate of algae	0.90 (0.12-1.9) *	d^{-1}	This study
μ_{bac}	Specific growth rate of bacteria	0.73 (0.70-0.76) *	d^{-1}	This study
Other kinetic parameters				
$K_{CO2,alg}$	Half saturation constant for algae growth on	0.035	mg C L^{-1}	(Keymer et al. 2013)
b_{alg}	Decay rate coefficient of algae	0.010	d^{-1}	(Reichert et al. 2001, Richmond
K_{LaO2}	Mass transfer coefficient of O_2	24	d^{-1}	Measured in this study
I_{opt}	Optimum irradiance	100	μmol photons m^{-2} s^{-1}	(Li et al. 2012)
Stoichiometric parameters				
k_1	Reaction rate for hydration of CO_2	1.0×10^4	d^{-1}	(Decostere et al. 2013)
k_2	Reaction rate for dissociation of HCO_3^-	1.0×10^4	d^{-1}	(Decostere et al. 2013)
$a_{C,alg}$	Mass fraction of carbon of algal biomass	0.522	mg C mg X_{alg} $^{-1}$	Measured in this study

* Bracket values show 95% confidence intervals.

4. Results and Discussion

Algal growth was monitored through cultivation (7 days for Batch 1 and 9 days for Batch 2) by measuring OD_{680} every 24 hours. The amount of algal biomass was calculated based on the linear regression method described in section 2.2. The data were normalized to ΔX_{alg}. ΔX_{alg} was calculated by $\Delta X_{alg} = X_{alg}(t) - X_{alg}(0)$, where $X_{alg}(t)$ is algal biomass concentration at cultivation time t, and $X_{alg}(0)$ is algal biomass concentration at the start of the experiment. Normalized algal biomass accumulation through cultivation time is shown in Figure 2. For both batches (pH 7.5 and 8.5), a significant increase in algal growth was observed by adding heterotrophic bacteria. After 7 days of cultivation, algal productivity was 4.8 and 3.4 times higher in the systems supplemented with bacteria. The stimulative effect of bacteria on algal growth has been described as being associated with bacteria mineralising carbon (Ask et al., 2009; Muñoz and Guieysse, 2006), as well as cycling nutrients (Bloesch et al., 1977; Zhao et al., 2012) and generating vitamins (Grant et al., 2014) and other growth promoting regulators. Considering inorganic carbon limitation, this work focuses on bacterial stimulation via mineralising photosynthetic end products. As Maeda *et al.* (1995) discussed in their study, *Chlorella* sp. suffers from CO_2 limitation with air (CO_2 0.038 % v v^{-1}) supply, while 10 % CO_2 supply dramatically boosts the growth of algae. Increasing CO_2 content in the air supply is a conventional method to eliminate the carbon restriction. The experimental results in this study suggest that carbon cycling by heterotrophic bacteria can achieve results similar to those seen for CO_2 supplementation.

In addition, it is apparent that algal productivity was higher at pH 7.5 than at pH 8.5. pH is an important component of the bicarbonate buffering system because it has a direct influence on the species of the inorganic carbon available to the algae. Increasing pH from 7.5 to 8.5 lowers the CO_2 pool from 6.7 % to 0.7 % of the total dissolved inorganic carbon (DIC). It is acknowledged that the majority of algae (all cyanobacteria and most of green eukaryotic algae) have developed carbon concentrating mechanisms (CCMs) as an evolutionary response to low CO_2 levels. For example, they can convert HCO_3^- to CO_2 by carbonic anhydrase activity, resulting in an internal Ci (internal inorganic carbon concentration) of 5 to even 75-fold higher than that of the surrounding medium (Wang et al., 2011). But it has been shown that the *Chlorella* sp. applied in this study has a negligible affinity for HCO_3^- as a growth substrate (Keymer et al., 2013). A possible explanation for this is the lack of carbonic anhydrase (CA) in this algae, preventing it from converting HCO_3^- to CO_2 (Rotatore and Colman, 1991a; Rotatore and Colman, 1991b). Thus the activity of the algae used in this study is

particularly sensitive to CO_2, which is why the activity was markedly higher at the lower pH.

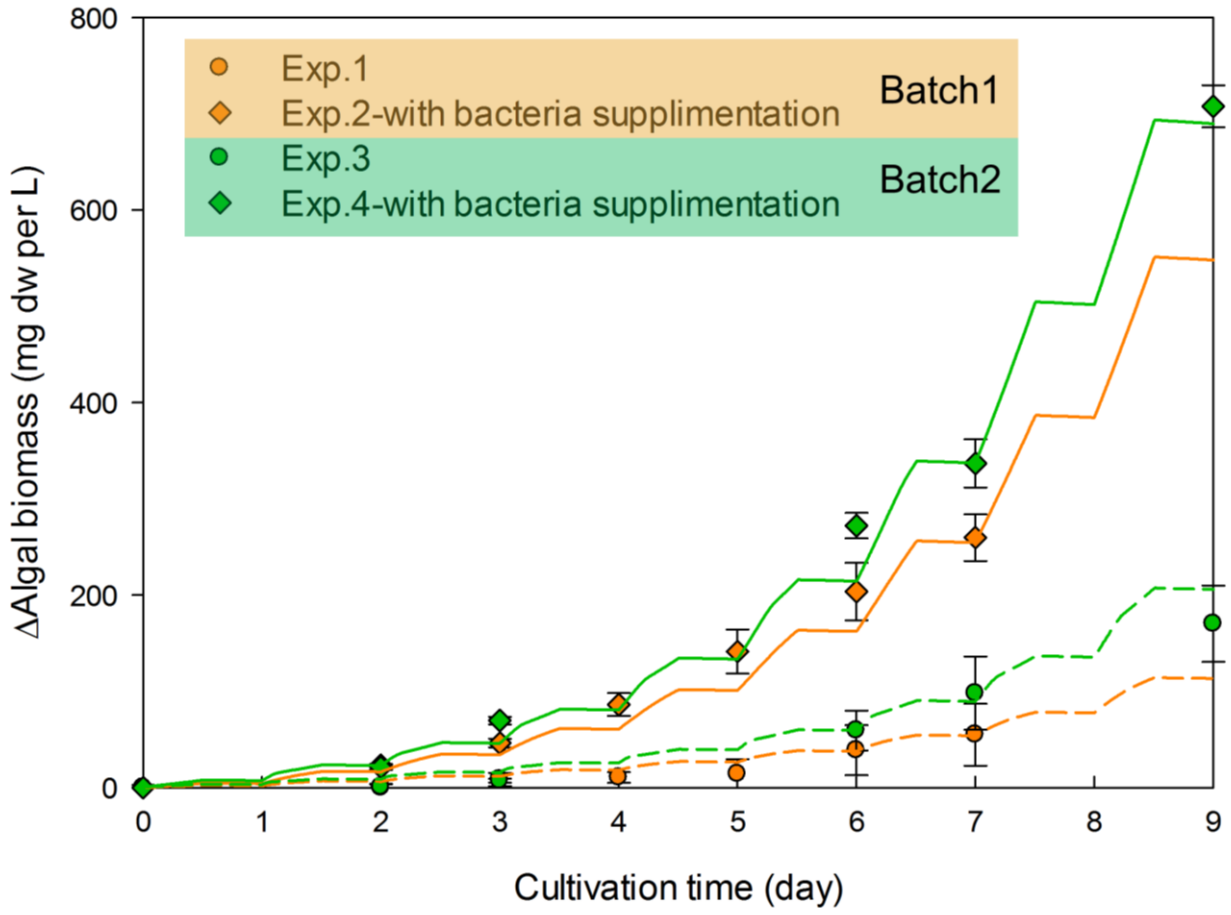


Figure 2. Experimental data (symbols) and model simulation results (lines) of algal productivity ΔX_{alg} Organge and green lines show the model simulation results of Batch 1 and Batch 2, respectively. Error bars show standard errors of Batch 1 (n=3) and Batch 2 (n=2), n is the number of samples.

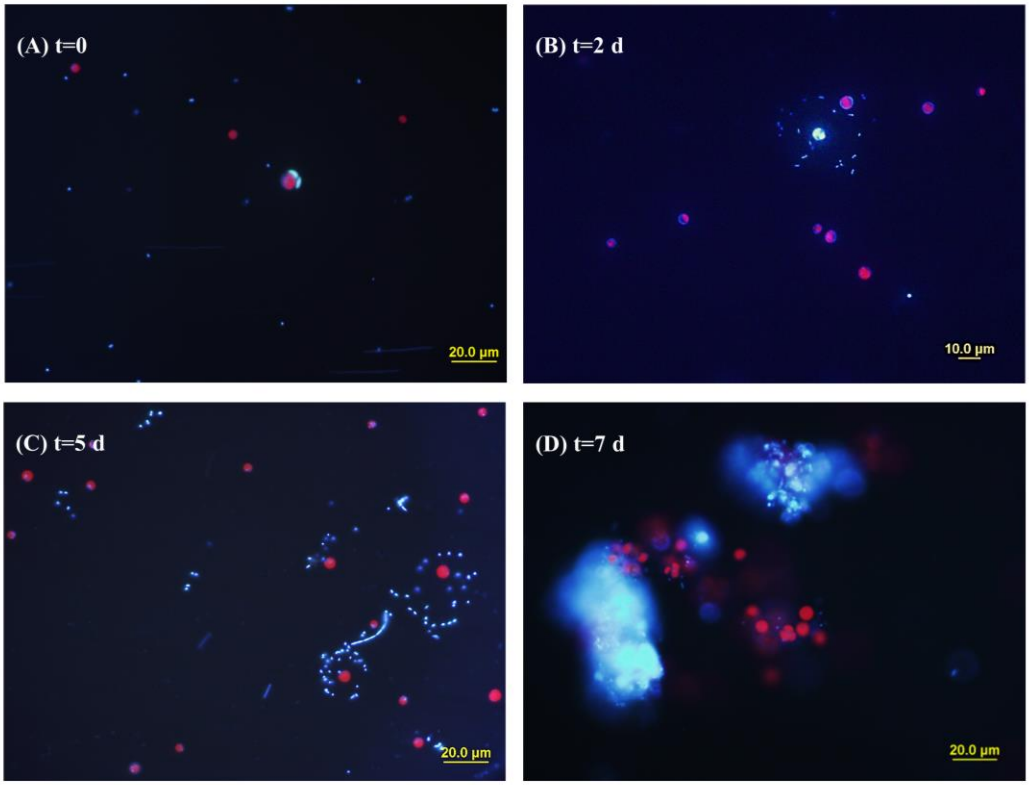


Figure 3. Flourescent microscope images of algal and bacteria cells from Exp.4 (Batch 2 with supplementary bacteria). (A) at the start of experiment, (B) at day 2, (C) at day 5 and (D) at day 7. Algae are flourescent red by chlorophyll autoflourescent. Bacteria are flourescent bright blue by UV excitation of DAPI stain.

Fluorescent microscope images (Figure 3) show the algal and bacterial cells from Exp. 4 through cultivation. After 7 days, the amount of algal cells increased, which was in line with the OD_{680} measurement results. Comparing Figure 3A and Figure 3D shows that a significant amount of bacterial growth occurred. Algal and bacterial cells were tending to form flocs in the culture through the co-cultivation. The natural bioflocculation process of algal and bacterial biomass has been reported by several studies and applied to some wastewater treatment processes (Gutzeit et al., 2005; Van Den Hende et al., 2011). A benefit of forming flocs is that bacterial cells may increase the local concentration of dissolved CO_2 for algal cells. Meanwhile, heterotrophic bacteria can grow on either the detritus of dead algal cells or on the extracellular polymeric substances (EPS) produced by the algal cells. A major conclusion from Oswald and Gotaas (1957) and Gutzeit et al. (2005) was that formation of flocs by unicellular algae and wastewater heterotrophic bacteria could benefit the

interactions between photoautotrophic algae and heterotrophic bacteria concerning the exchange of O_2 and CO_2.

The model results shown in Figure 2 were obtained using the model described in Table I. The model parameters are shown in Table III. The key growth rates, $\mu_{max,alg}$ and μ_{bac}, were determined by least squares parameter estimation using experimental datasets from Batch 1. The estimated maximum specific growth rate of algae, $\mu_{max,alg}$, was 0.90 d^{-1}, which is consistent with the rate reported by Keymer (2013) for the same species. This maximum specific growth rate, applied to the dilute initial condition, resulted in the production of approximately 298 mg L^{-1} of algae in the bacteria-supplemented system but only about 76 mg L^{-1} of algae in the control (for a 7 day of cultivation period). The estimated bacterial net growth rate, μ_{bac}, was 0.73 d^{-1}. This lumped term can be expressed as $\mu_{max,bac}\frac{S_S}{S_S+K_{SS,bac}}\frac{S_{O2}}{S_{O2}+K_{O2,bac}}$. As reported in the literature, $\mu_{max,bac}$ generally lies between 5-10 d^{-1} (Henze et al. 2000). DO was monitored during the experiments, and varied from lowest 7.75 mg O_2 L^{-1} at night to a maximum of 18.58 mg O_2 L^{-1} at midday. Therefore the DO in the system was sufficient for bacterial growth (Stenstrom and Song, 1991). A possible explanation for the low μ_{bac} was a low concentration of organic substrates (S_s). The organic substrates for bacterial growth were mainly from by-products of algae growth and metabolism, such as detritus of dead algae or EPS (Process 3 of Figure 1). It is expected that the amount of these substrates is lower than the half saturation constant for bacterial growth on organic substrates.

The calibrated model, using the parameters estimated from Batch 1, was then applied to simulate Batch 2. Simulated increase in algal biomass concentration correlated very well with the measured increase in algal biomass concentration (R^2 of 0.99).

Figure 4 was plotted to give an overview of what one may expect for a range of pH values and the effect of the presence of bacteria in the culture system. When bacteria is not present in the culture (Figure 4A), a plateau appears at 0.8 ($\log_{10}$ mg DW L^{-1} d^{-1}), indicating a limitation of the algal biomass productivity by S_{CO2}. However, when bacteria are supplemented in the system (Figure 4B), this limitation is no longer present due to bacteria recycling carbon and making it available for algal growth.

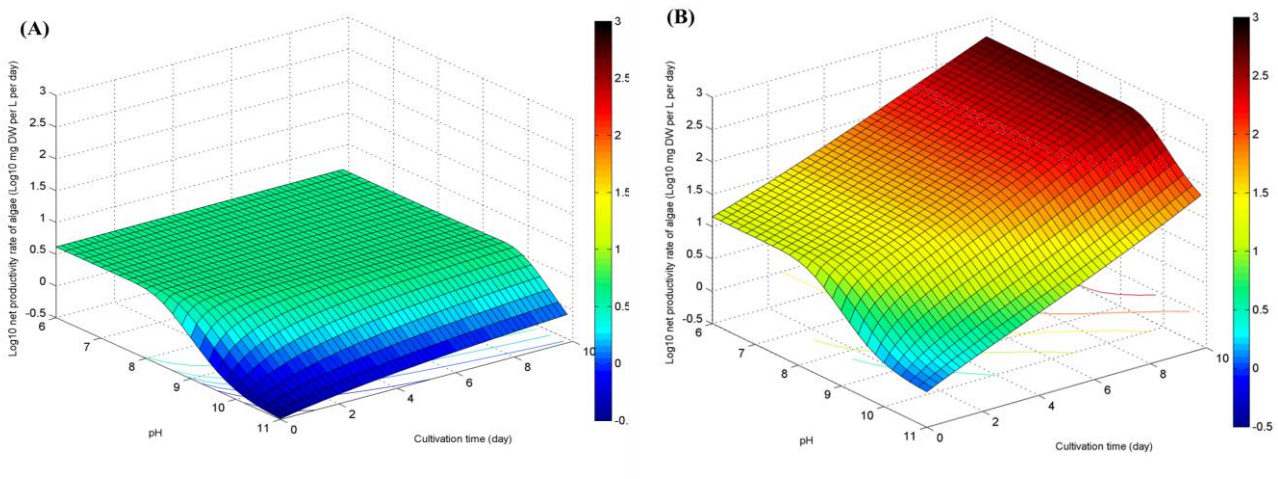


Figure 4. Example surface plots showing the model simulation results over a range of pH values using initial conditions measured and parameters estimated from this study: (A) without bacteria supplementation; (B) with bacteria supplementation.

5. Conclusions

Adding heterotrophic bacteria to an open algal culture dramatically enhances algal growth. It is likely that this is at least in part due to bacteria cycling carbon by re-mineralising photosynthetic end products. In this work we modelled the contribution of bacteria and showed that supplementing bacteria to an open algal system can overcome the inorganic carbon limitation that would be expected in an air supplied algal culture.

Nomenclature

Abbreviations

CA carbon anhydrase
CCMs carbon concentrating mechanisms
DIC dissolved inorganic carbon (mg C L^{-1})
DO dissolved oxygen (mg O_2 L^{-1})
DW dry weight
EPS extracellular polymeric substances
HRAP high rate algal pond
OD_{680} optical density at wavelength 680 nm
TIC total inorganic carbon (mg C L^{-1})

Symbols

a	light absorption coefficient on a dry weight basis (m^2 mg^{-1})
$a_{C,alg}$	mass fraction of carbon of algal biomass (mg C mg X_{alg} $^{-1}$)
b_{alg}	decay rate coefficient of algae (d^{-1})
I_{ave}	average irradiance (µmol photons m^{-2} s^{-1})
I_0	incident irradiance (µmol photons m^{-2} s^{-1})
I_{opt}	optimum irradiance (µmol photons m^{-2} s^{-1})
$k1$	reaction rate for hydration of CO_2 (d^{-1})
$k2$	reaction rate dissociation of HCO_3^- (d^{-1})
K_{eq1}	equilibrium constant of CO_2 toHCO_3^-
K_{eq2}	equilibrium constant of HCO_3^- to CO_3^{2-}
$K_{CO2,alg}$	half saturation constant for algae growth on CO_2 (mg C L^{-1})
$K_{SS,bac}$	half saturation constant for bacteria growth on S_S (mg L^{-1})
$K_{O2,bac}$	half saturation constant for bacteria growth on O_2 (mg O_2 L^{-1})
K_{LaO2}	mass transfer coefficient of O_2 (d^{-1})
K_{LaCO2}	mass transfer efficiency of CO_2 (d^{-1})
r_j	process rate expressions
R_{alg}	algal biomass productivity (mg DW L^{-1} d^{-1})
S_{CO2}	dissolved CO_2 concentration (mg C L^{-1})
S_{CO2}^*	saturated dissolved CO_2 concentration (mg C L^{-1})
S_{CO3}	dissolved CO_3^{2-} measured as carbon mass (mg C L^{-1})
S_H	hydrogen ions (mmole H L^{-1})
S_{HCO3}	dissolved HCO_3^- measured as carbon mass (mg C L^{-1})
S_{O2}	dissolved O_2 (mg O_2 L^{-1})
S_S	organic substrates (mg L^{-1})
μ_{bac}	net growth rate of bacteria (d^{-1})
$\mu_{max,alg}$	maximum specific growth rate of algae (d^{-1})
$\mu_{max,bac}$	maximum specific growth rate of bacteria (d^{-1})
ϑ_{ij}	stoichiometric coefficients
X_{alg}	algal biomass (mg L^{-1})
ΔX_{alg}	normalized algal biomass concentration (mg L^{-1})
$X_{alg}(0)$	algal biomass concentration at the start of the experiment (mg DW L^{-1})
$X_{alg}(t)$	algal biomass concentration at cultivation time t (mg DW L^{-1})
X_{bac}	bacterial biomass (mg L^{-1})
z	reactor light path (m)

Reference

Andersen RA. 2005. Algal culturing techniques. Boston, Mass: Elsevier/Academic Press.

Ask J, Karlsson J, Persson L, Ask P, Byström P, Jansson M. 2009. Whole-lake estimates of carbon flux through algae and bacteria in benthic and pelagic habitats of clear-water lakes. Ecology 90:1923-1932.

Azov Y, Goldman JC. 1982. Free ammonia inhibition of algal photosynthesis in intensive cultures. Appl Environ Microb 43:735-739.

Barbosa MJ, Hoogakker J, Wijffels RH. 2003. Optimisation of cultivation parameters in photobioreactors for microalgae cultivation using the A-stat technique. Biomol Eng 20:115-123.

Bloesch J, Stadelmann P, Buhrer H. 1977. Primary production, mineralization, and sedimentation in the euphotic zone of two Swiss lakes. Limnol. Oceanogr 22:1.

Brennan L, Owende P. 2010. Biofuels from microalgae—A review of technologies for production, processing, and extractions of biofuels and co-products. Renewable and Sustainable Energy Reviews 14:557-577.

Buhr HO, Miller SB. 1983. A dynamic model of the high-rate algal-bacterial wastewater treatment pond. Water Res 17:29-37.

Cole JJ. 1982. Interactions Between Bacteria and Algae in Aquatic Ecosystems. Annu Rev Ecol Syst 13:291-314.

Chisti Y. 2007. Biodiesel from microalgae. Biotechnol Adv 25:294-306.

Fukami K, Nishijima T, Ishida Y. 1997. Stimulative and inhibitory effects of bacteria on the growth of microalgae. Hydrobiologia 358:185-191.

Gallagher BJ. 2011. The economics of producing biodiesel from algae. Renew Energ 36:158-162.

Grant MA, Kazamia E, Cicuta P, Smith AG. 2014. Direct exchange of vitamin B12 is demonstrated by modelling the growth dynamics of algal–bacterial cocultures. The ISME journal: 1-10. Doi: 10.1038/ismej.2014.9.

Gutzeit G, Lorch D, Weber A, Engels M, Neis U. 2005. Bioflocculent algal-bacterial biomass improves low-cost wastewater treatment. Water science and technology: a journal of the International Association on Water Pollution Research 52:9.

Ho S-H, Chen W-M, Chang J-S. 2010. Scenedesmus obliquus CNW-N as a potential candidate for CO2 mitigation and biodiesel production. Bioresour Technol 101:8725-8730.

Huisman J. 1999. Population dynamics of light-limited phytoplankton: microcosm experiments. Ecology 80:202-210.

Jupsin H, Praet E, Vasel J. 2003. Dynamic mathematical model of high rate algal ponds (HRAP). Water science and technology: a journal of the International Association on Water Pollution Research 48(2):197.

Keymer P, Lant P, Pratt S. 2013. Modelling microalgal activity as a function of inorganic carbon concentration: accounting for the impact of pH on the bicarbonate system. J Appl Phycol:1-8.

Li Y, Zhou W, Hu B, Min M, Chen P, Ruan RR. 2012. Effect of light intensity on algal biomass accumulation and biodiesel production for mixotrophic strains Chlorella kessleri and Chlorella protothecoide cultivated in highly concentrated municipal wastewater. Biotechnol Bioeng 109:2222-2229.

Maeda K, Owada M, Kimura N, Omata K, Karube I. 1995. CO2 fixation from the flue gas on coal-fired thermal power plant by microalgae. Energ Convers Manage 36:717-720.

Muñoz R, Guieysse B. 2006. Algal-bacterial processes for the treatment of hazardous contaminants: A review. Water Res 40:2799-2815.

Oswald WJ, Gotaas HB. 1957. Photosynthesis in sewage treatment. Trans. Am. Soc. Civ. Eng 122:73-105.

Porter KG, Feig YS. 1980. The use of DAPI for identifying and counting aquatic microflora. Limnol Oceanogr:943-948.

Reichert P. 1998. Aquasim 2.0—User Manual, Computer Program for the Identification and Simulation of Aquatic Systems.

Reichert P, Borchardt D, Somlyódy L, Shanahan P, Rauch W, Vanrolleghem P, Henze M. 2001. River Water Quality Model no. 1 (RWQM1): II. Biochemical process equations. Water science and technology : a journal of the International Association on Water Pollution Research 43:11-30.

Richmond A. 1992. Open systems for the mass production of photoautotrophic microalgae outdoors: physiological principles. J Appl Phycol 4:281-286.

Riquelme CE, Fukami K, Ishida Y. 1987. Annual Fluctuations of phytoplankton and bacterial communities in Maizuru bay and their interrelationship. Bulletin of Japanese Society of Microbial Ecology 2:29-37.

Rotatore C, Colman B. 1991a. The active uptake of carbon dioxide by the unicellular green algae Chlorella saccharophila and C. ellipsoidea. Plant, Cell & Environment 14:371-375.

Rotatore C, Colman B. 1991b. The localization of active inorganic carbon transport at the plasma membrane in Chlorella ellipsoidea. Canadian Journal of Botany 69:1025-1031.

Sander R. 1999. Compilation of Henry's law constants for inorganic and organic species of potential importance in environmental chemistry. Max-Planck Institute of Chemistry, Air Chemistry Department.

Stenstrom MK, Song SS. 1991. Effects of Oxygen Transport Limitation on Nitrification in the Activated Sludge Process. Research Journal of the Water Pollution Control Federation 63:208-219.

Suminto, Hirayama K. 1997. Application of a growth-promoting bacteria for stable mass culture of three marine microalgae. Hydrobiologia 358:223-230.

Van Den Hende S, Vervaeren H, Saveyn H, Maes G, Boon N. 2011. Microalgal bacterial floc properties are improved by a balanced inorganic/organic carbon ratio. Biotechnol Bioeng 108:549-558.

Van Loosdrecht MCM, Henze M. 1999. Maintenance, endogeneous respiration, lysis, decay and predation. Water Sci Technol 39:107-117.

Wang Y, Duanmu D, Spalding MH. 2011. Carbon dioxide concentrating mechanism in Chlamydomonas reinhardtii: inorganic carbon transport and CO2 recapture. Photosynth Res 109:115-122.

Yang A. 2011. Modeling and Evaluation of CO2 Supply and Utilization in Algal Ponds. Ind Eng Chem Res 50:11181-11192.

Zhao B, Su Y. 2014. Process effect of microalgal-carbon dioxide fixation and biomass production: A review. Renewable and Sustainable Energy Reviews 31:121-132.

Zhao G, Du J, Jia Y, Lv Y, Han G, Tian X. 2012. The importance of bacteria in promoting algal growth in eutrophic lakes with limited available phosphorus. Ecol Eng 42:107-111.

Zhao, G., Du, J., Jia, Y., Lv, Y., Han, G., Tian, X. 2012. The importance of bacteria in promoting algal growth in eutrophic lakes with limited available phosphorus. Ecol. Eng. 42:107-111.

Supporting Information

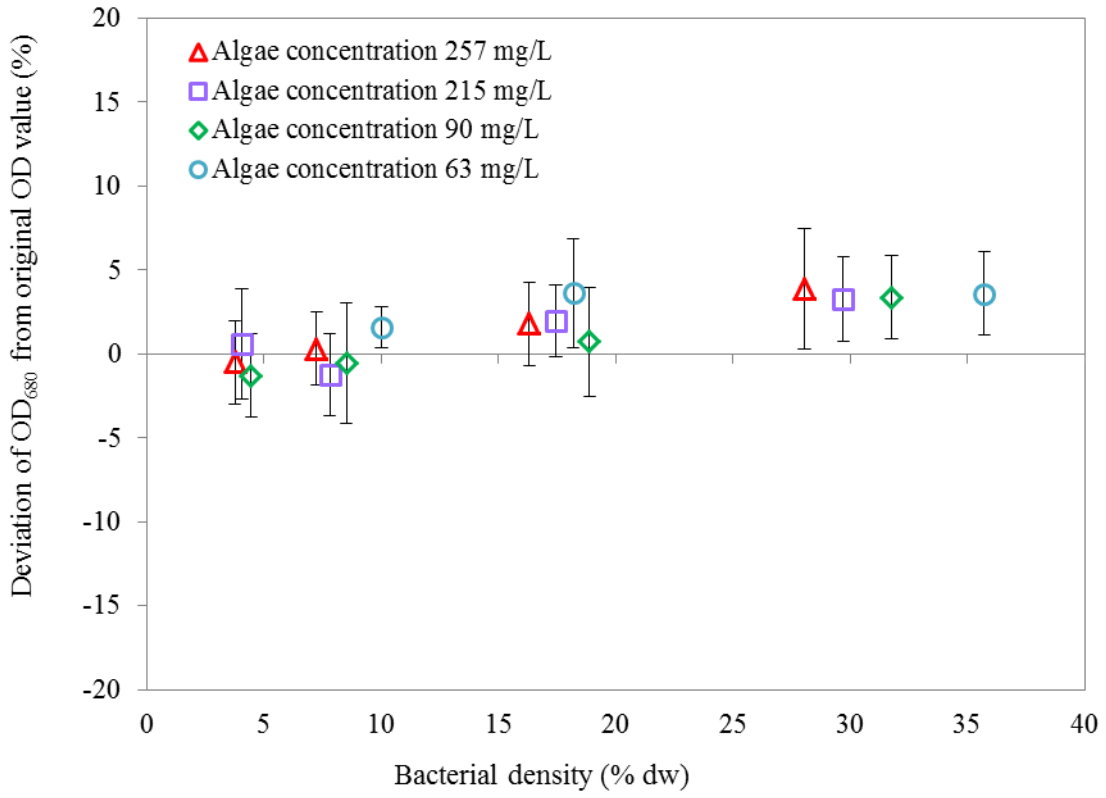


Figure S1. Verification of the interference from bacterial density to the OD680 measurement of algal biomass within the time frame of this study.

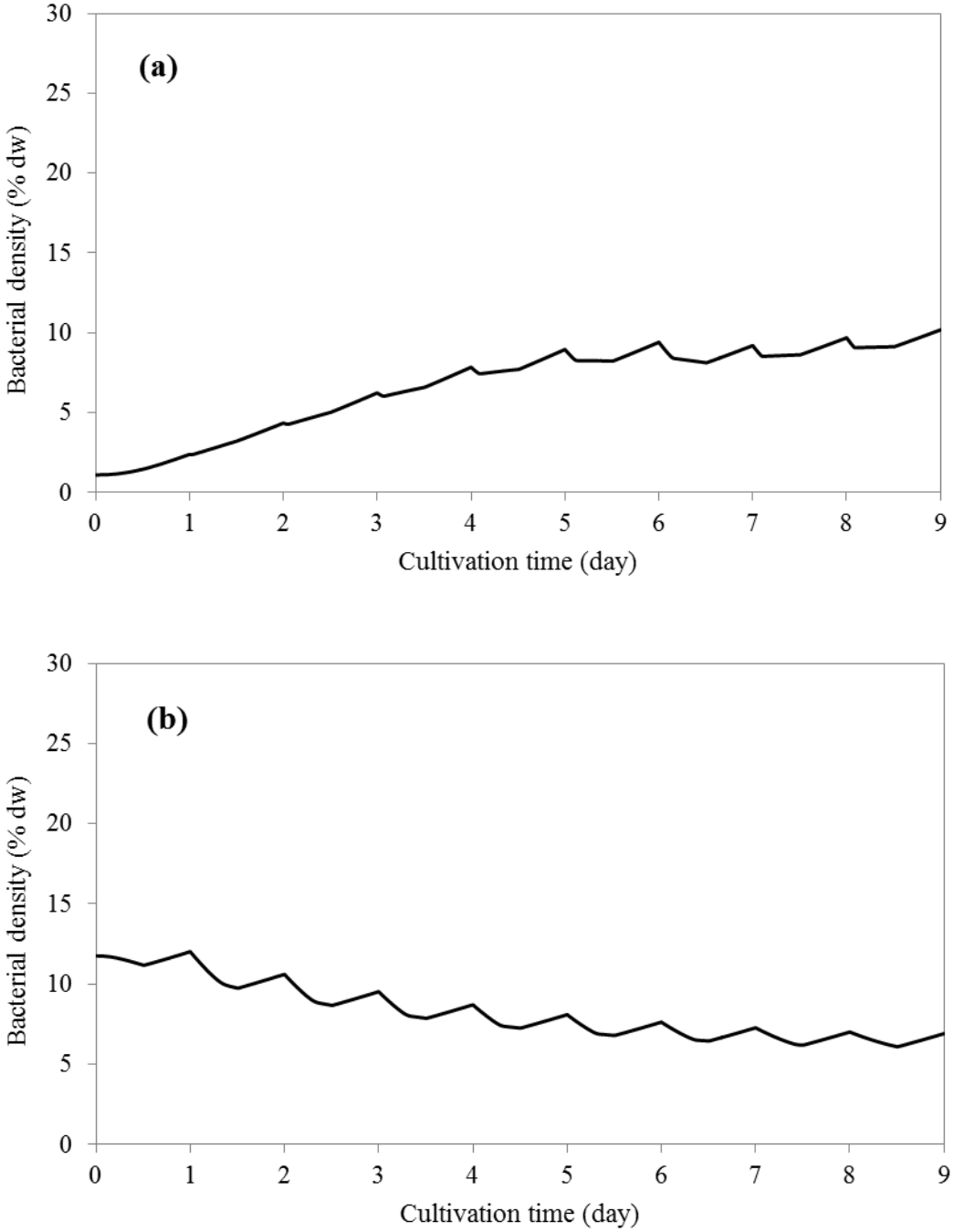


Figure S2. The simulation result of bacteria density through 9 days cultivation (a) control (Exp.3), (b) bacteria supplemented system and (Exp.4).

Appendix B

Algae-Bacteria Model (ABM) for algal mass cultivation and process control

1 Model Format—Petersen's Matrix

Simulation of open algal mass cultivation systems, incorporating phenomena such as photosynthesis and heterotrophic growth of biomass, must necessarily account for a large number of reactions between large numbers of components. To be mathematically tractable while providing realistic predictions, the reactions must be representative of the most important fundamental processes occurring within the system. One problem often associated with papers presenting models describing complex systems is that it is difficult to follow the development of the author's ideas. In particular, it is often difficult to trace all the interactions of the system components. Here we concluded that a matrix format, based on the work of Petersen (1965), for presentation of the model offered the best opportunity for overcoming this problem while conveying the maximum amount of information.

The advantages of using Petersen's Matrix format to represent ABM are as follows,

- Offering a clear approach to convey the maximum amount of the information in AMB.
- Offering a convenient way to adding or omitting biological or chemical processes in the model to simulate a new operating mode.

2 Simplifying assumptions and description illumination

The ABM is presented in a matrix format in Table 1. Because different units are used to characterize organic material, conversion formulas are given between mass of organic substances (OM; this is dry mass for particulate substances), organic carbon (OC) and chemical oxygen demand (COD). Since the composition of organic matter is approximated by mass fractions of the elements C, H, O, and N, and the mass fractions of all other elements are neglected, the composition of organic material can uniquely be described by the mass fractions of C, H, O, and N (Eq. 1), where a mass fraction represents the fraction of the total mass of organic substance contributed by a particular chemical element.

$$\alpha_C, \alpha_H, \alpha_O, \alpha_N, \quad \text{Eq. 1}$$

Because other elements are neglected, these mass fractions fulfil the constraint

$$\alpha_C + \alpha_H + \alpha_O + \alpha_N = 1 \quad \text{Eq. 2}$$

The mass fractions make a formulation of organic matter by a chemical formula possible. For 1 g of organic matter this formula is given as (indices are interpreted as fractions of moles)

$$C_{\alpha_{C/12}} H_{\alpha_H} O_{\alpha_{O/16}} N_{\alpha_{N/14}} \quad \text{Eq. 3}$$

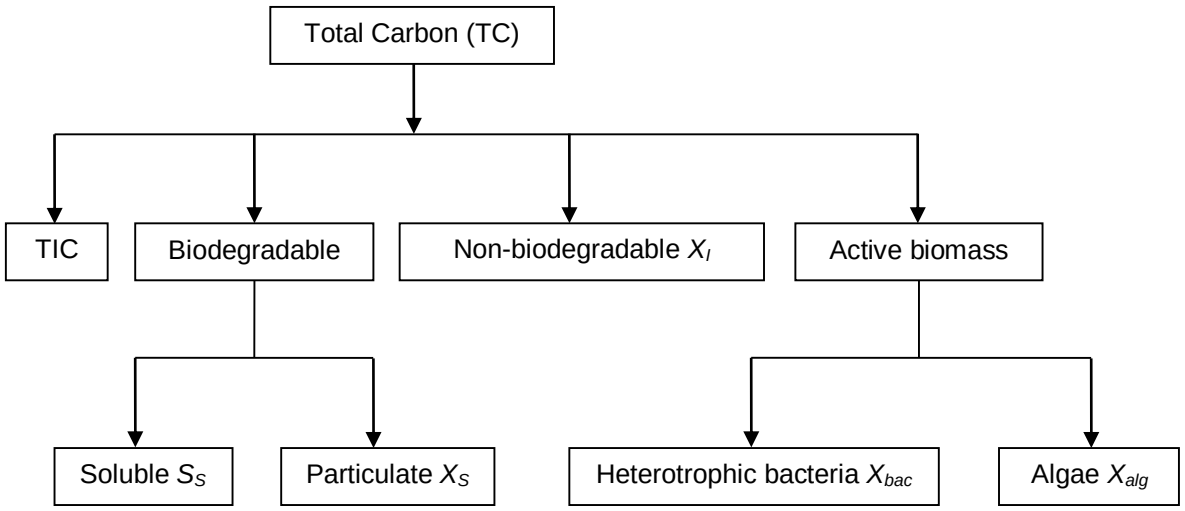


Figure 1 Total carbon components in the algae-bacteria model.

Carbon components in algae-bacteria model

In order to trace the carbon flow, total carbon is subdivided based on (i) solubility, (ii) biodegradability (iii) biodegradation rate, and (iv) viability (biomass) (as shown in Figure 1). Table 2 summarizes all the state variables applied in the model.

(i) The total carbon is divided into soluble (S) and particulate (X) components.
(ii) The total carbon is further subdivided into non-biodegradable organic matter (X_I) and biodegradable matter (X_S, S_S).
(iii) The biodegradable matter is divided into soluble readily biodegradable (S_S) and slowly biodegradable (X_S) substrate.
(iv) Finally, heterotrophic bacterial biomass (X_{bac}) and algal biomass (X_{alg}) are generated by growth on the readily biodegradable substrate (S_S) and inorganic carbon (TIC) respectively.

Table 1: Stoichiometric matrix for the established Algae-Bacteria Model.

	Component i →	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**Rates**
	Process j ↓	S_S	S_{CO2}	S_{HCO3}	S_{CO3}	S_{O2}	S_{NO3}	S_{NH4}	S_H	S_{OH}	X_{alg}	X_{bac}	X_S	X_I	
1	Growth of algae with CO_2		-0.52			1.62	-0.026		-0.0018		1.00				$\mu_{max,alg}\frac{S_{CO2}}{S_{CO2}+K_{CO2,alg}}\frac{S_{NO3}}{S_{NO3}+K_{NO3,alg}}\frac{I_{ave}}{I_{opt}}e^{(1-\frac{I_{ave}}{I_{opt}})}X_{alg}$
2	Respiration of algae		0.40			-1.49		0.02	0.01		-1.00			0.20	$k_{resp,alg}\frac{S_{O2}}{K_{O2,alg}+S_{O2}}X_{alg}$
3	Death of algae		0.16			-0.40		-0.01	0.00		-1.00		0.50	0.12	$b_{alg}X_{alg}$
4	Growth of bacteria	-1.67	0.43			-1.39		-0.0033	0.00024			1.00			$\mu_{max,bac}\frac{S_S}{S_S+K_S}\frac{S_{NH4,bac}}{S_{NH4,bac}+K_{NH4,bac}}\frac{S_{O2}}{S_{O2}+K_{O2,bac}}X_{bac}$
5	Respiration of bacteria		0.40			-1.59		0.11	0.01			-1.00		0.20	$k_{resp,bac}\frac{S_{O2}}{S_{O2}+K_{O2,bac}}X_{bac}$
6	Death of bacteria		0.16			-0.44		0.08	-0.01			-1.00	0.50	0.12	$b_{bac}X_{bac}$
7	Hydrolysis	1.00											-1.00		$k_{hyd}X_S$
8	Equilibrium $H_2O \rightarrow H^+ + OH^-$								1.00	1.00					$1 - S_H S_{OH}/K_{eq,w}$
9	Equilibrium $CO_2 \rightarrow HCO_3^-$		-1.00	1.00											$k1 \times \left(S_{CO2} - S_H \times \frac{S_{HCO3}}{K_{eq1}}\right)$
10	Equilibrium $HCO_3^- \rightarrow CO_3^{2-}$			-1.00	1.00										$k2 \times \left(S_{HCO3} - S_H \times \frac{S_{CO3}}{K_{eq2}}\right)$

11	Equilibrium $NH_4^+ \rightarrow NH_3$			$S_{NH4} - S_{NH3}S_H/K_{eq3}$
12	Gas-liquid mass transfer of O_2		1.00	$K_{LaCO2}(S^*_{CO2} - S_{CO2})$
13	Gas-liquid mass transfer of CO_2	1.00		$K_{LaO2}(S^*_{O2} - S_{O2})$

Table 2: State variables.

Symbol	Description	Unit
$\boldsymbol{X_{alg}}$	Algal biomass.	mg X_{alg} L^{-1}
$\boldsymbol{X_{bac}}$	Bacterial biomass.	mg X_{bac} L^{-1}
$\boldsymbol{X_S}$	Particulate organic material, assumed to be available for biodegradation after hydrolysis. These substances must undergo hydrolysis catalyzed by heterotrophic organisms before being directly degradable.	mg X_S L^{-1}
$\boldsymbol{X_I}$	Inert particulate organic material. These substances are assumed to be not biodegradable within the time frame of relevance.	mg X_I L^{-1}
$\boldsymbol{S_S}$	Dissolved organic substances, assumed to be available for rapid biodegradation by heterotrophic bacteria.	mg S_S L^{-1}
$\boldsymbol{S_{CO2}}$	Sum of dissolved carbon dioxide and H_2CO_3 measured as carbon mass: CO_2—C+H_2CO_3—C.	mg C L^{-1}
$\boldsymbol{S_{HCO3}}$	Dissolved HCO_3^- measured as carbon mass: HCO_3^-—C.	mg C L^{-1}
$\boldsymbol{S_{CO3}}$	Dissolved CO_3^{2-} measured as carbon mass: CO_3^{2-}—C.	mg C L^{-1}
$\boldsymbol{S_{O2}}$	Dissolved oxygen: O_2.	mg O_2 L^{-1}
$\boldsymbol{S_{NO3}}$	Nitrate-nitrogen: NO_3^-—N.	mg N L^{-1}
$\boldsymbol{S_{NH4}}$	Ammonium nitrogen: NH_4^+—N.	mg N L^{-1}
$\boldsymbol{S_{NH3}}$	Ammonia nitrogen: NH_3^-—N.	mg N L^{-1}
$\boldsymbol{S_H}$	Hydrogen ions: H^+.	mmole H L^{-1}
$\boldsymbol{S_{OH}}$	OH- ions measured as hydrogen mass (or moles): OH^-—H.	mmole OH L^{-1}

As shown in Figure 2, the following processes are considered in the model.

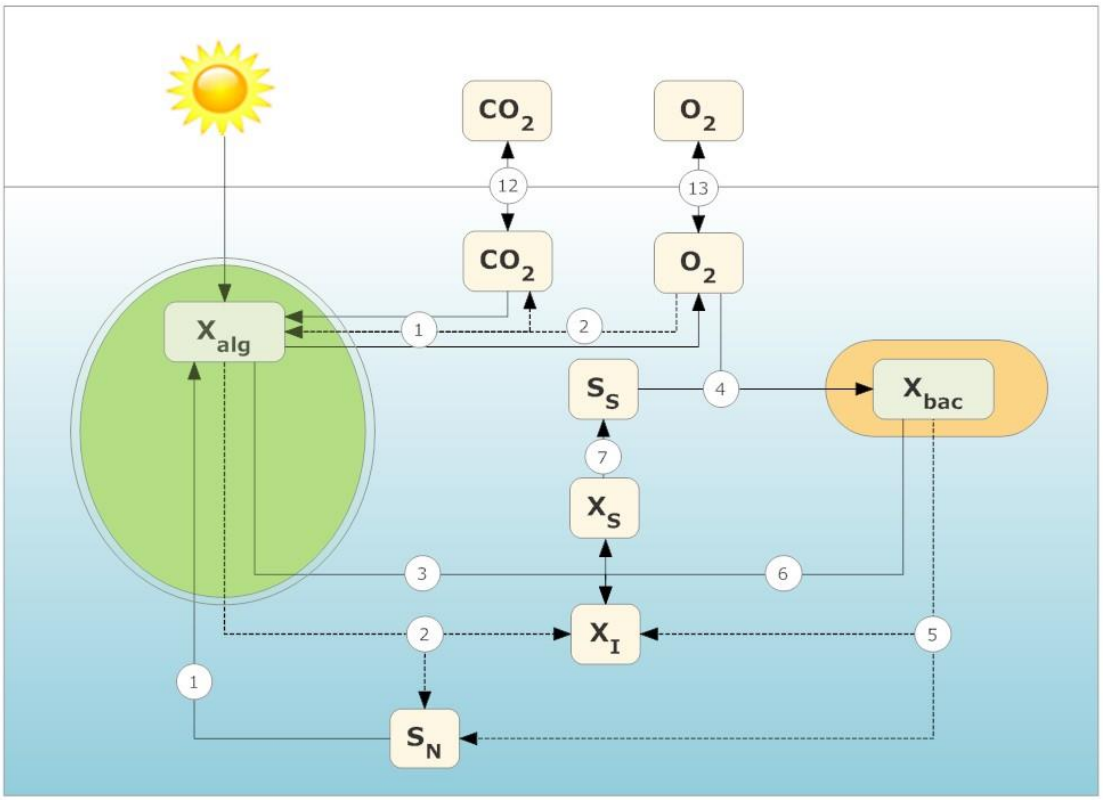


Figure 2 Carbon flows in algae-bacteria model. Numbers in the model represent biochemical processes.

Process 1: Autotrophic growth of algae with NO_3^-

Growth of algae (X_{alg}) is the growth of algae by photosynthesis. The rate of algal photosynthesis mainly depends on several limiting factors: light, inorganic carbon source, other limiting nutrients (nitrogen and phosphorus for all algal species, silicon for diatom, and so on), and temperature. Different types of algal growth model have been developed by researchers and engineers since 1960. Basic algal growth and its kinetic expression are described by using many types of mathematical equations. Most widely discussed models are Monod equation (Grima et al., 1994; Holmberg, 1982), Haldane equation (Chisti, 2007), and Steel equation (Baquerisse et al., 1999; Benson et al., 2007).

Considering light saturation and light limitation for algae growth, the Steel equation is widely used (Eq. 4). The Lambert-Beer law is used in order to incorporate the depth of the reactor and self-shading, as shown in Figure 3. It is assumed that as light travels through the reactor, its rate of absorption by phytoplankton is analogous to attenuation described by the Lambert-Beer model (Huisman, 1999). At a depth of z m, the irradiance is related to the incident irradiance I_0 by the function $I(z) =$

$I_0 exp(-a(\lambda)X_{alg}z)$, where X_{alg} is the algae density (mg L^{-1}) and $a(\lambda)$ is the absorption coefficient of the algae (m^2 g^{-1}). This model assumes that the biomass density is homogenous due to mixing.

$$\mu = \mu_{max} \frac{I_{ave}}{I_{opt}} e^{(1-\frac{I_{ave}}{I_{opt}})} \quad \text{Eq. 4}$$

In order to find the average light intensity, integration over the path length inside the reactor yields:

$$I_{out} = I_0 \cdot e^{-a(\lambda)\cdot X_{alg}\cdot z} \quad \text{Eq. 5}$$

$$I_{ave} = \frac{\int_0^z I_0 \cdot e^{-a(\lambda)\cdot X_{alg}\cdot x}\, dx}{\int_0^z 1\, dx} \quad \text{Eq. 6}$$

$$I_{ave} = I_0 \cdot \frac{1}{z}\left(1 - e^{-a(\lambda)\cdot X_{alg}\cdot z}\right) \cdot \frac{1}{X_{alg} a(\lambda)} \quad \text{Eq. 7}$$

where z is reactor light path and $a(\lambda)$ is spectral-averaged absorption coefficient on a dry weight basis(Barbosa et al., 2003).

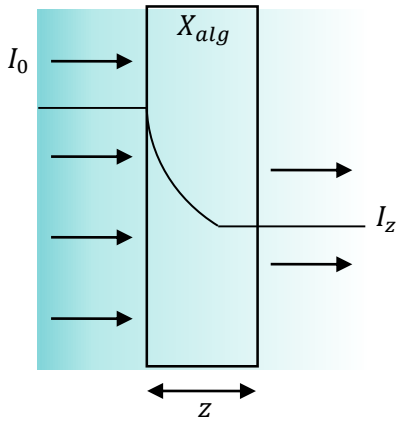


Figure 3 Light attenuation in photobioreactor.

Process 4: Aerobic growth of heterotrophic bacteria

Growth of heterotrophic bacteria using dissolved organic substrate, dissolved oxygen, and nutrients. And the limiting factors to aerobic growth of heterotrophic bacteria in this model are assumed to be oxygen, nitrogen and organic substances; phosphate is assumed to be non-limiting in this model.

Processes 2, 5: Aerobic endogenous respiration of algae and bacteria

The product of aerobic degradates respiration is CO_2. Researchers start to investigate respiration of algae since 1970s (Burris, 1977). Geider and Osborne (1989) reviewed the quantitative relationship between dark respiration and growth of microalgae. They reported in their work that, under optimal conditions, respiration rates of algae are about 20-30% of growth rates, but the ratio of respiration to growth increases under suboptimal conditions (Zou et al., 2011). Aerobic respiration of bacteria is the process of loss of biomass by aerobic endogenous respiration.

Processes 3, 6: Decay of algae and bacteria

Decay of algae is the process of conversion of microalgal and bacterial biomass (X_{alg} and X_{bac}) to slowly degradable (X_S) and inert organic matter (X_I) by decay, lysis, *etc.* (Fong et al., 1994; Van Loosdrecht & Henze, 1999). In this process, a yield coefficient for the decay process is used to make mass conservation of all elements possible without requiring an uptake of oxygen, nitrogen, phosphorus or carbon during the decay process.

Process 7: Hydrolysis

Hydrolysis is the process of dissolution of slowly biodegradable particulate organic matter (X_S) to dissolved organic matter (S_S) catalysed by heterotrophic biomass. Similarly to the decay processes, a yield coefficient is introduced to guarantee that no oxygen, ammonia, or phosphate must be consumed during the hydrolysis process. If there is not strong evidence that the composition of particulate and dissolved organic matter is different, the same composition should be used and the yield coefficient set equal to unity.

Processes 8-11: Chemical equilibria (Not shown in Figure 2)

Chemical equilibria need to be considered in an open algae-bacteria culture system include the equilibria between CO_2 and HCO_3^-, between HCO_3^- and CO_3^{2-}, between NH_4^+ and NH_3, between H_2O and H^+ and OH^-.

Processes 12, 13: Gas-liquid mass transfer of carbon dioxide and oxygen

Carbon dioxide and oxygen gas-liquid mass transfer need to be considered in an open algae-bacteria culture system, which is controlled by mass transfer coefficient K_{La}.

Table 3: Stoichiometric coefficients of the ABM.

Subst.	Value	Unit
1	***Autotrophic growth of algae***	
$\mathbf{X_{alg}}$	1	mg X_{alg} L^{-1}
$\mathbf{S_{CO2}}$	$-\alpha_{C,alg}$	mg C L^{-1}
$\mathbf{S_{O2}}$	$\frac{8}{3}\alpha_{C,alg} + 8\alpha_{H,alg} - \alpha_{O,alg} + \frac{20}{7}\alpha_{N,alg}$	mg O_2 L^{-1}
$\mathbf{S_{NO3}}$	$-\alpha_{N,alg}$	mg N L^{-1}
$\mathbf{S_H}$	$-\frac{1}{14}\alpha_{N,alg}$	mmole H L^{-1}
2	***Aerobic endogenous respiration of algae***	
$\mathbf{S_{O2}}$	$-\frac{8}{3}(\alpha_{C,alg} - f_{I,alg}\alpha_{C,XI}) - 8(\alpha_{H,alg} - f_{I,alg}\alpha_{H,XI}) + (\alpha_{O,alg} - f_{I,alg}\alpha_{O,XI}) + \frac{12}{7}(\alpha_{N,alg} - f_{I,alg}\alpha_{N,XI})$	mg O_2 L^{-1}
$\mathbf{S_{CO2}}$	$\alpha_{C,alg} - f_{I,alg}\alpha_{C,XI}$	mg C L^{-1}
$\mathbf{S_{NH4}}$	$\alpha_{N,alg} f_{I,alg}\alpha_{N,XI}$	mg N L^{-1}
$\mathbf{S_H}$	$-\frac{1}{14}(\alpha_{H,alg} - f_{I,alg}\alpha_{H,XI})$	mmole H L^{-1}
$\mathbf{X_{alg}}$	-1	mg X_{alg} L^{-1}
$\mathbf{X_I}$	$f_{I,alg}$	mg X_I L^{-1}
3	***Death of algae***	
$\mathbf{S_{O2}}$	$(\alpha_{O,alg} - (1 - f_{I,alg})Y_{alg,death}\alpha_{O,XS} - f_{I,alg}Y_{alg,death}\alpha_{O,XI}) - 8(\alpha_{H,alg} - (1 - f_{I,alg})Y_{alg,death}\alpha_{H,XS} - f_{I,alg}Y_{alg,death}\alpha_{H,XI}) - \frac{8}{3}(\alpha_{C,alg} - (1 - f_{I,alg})Y_{alg,death}\alpha_{C,XS} - f_{I,alg}Y_{alg,death}\alpha_{C,XI}) + \frac{12}{7}(\alpha_{N,alg} - (1 - f_{I,alg})Y_{alg,death}\alpha_{N,XS} - f_{I,alg}Y_{alg,death}\alpha_{N,XI})$	mg O_2 L^{-1}
$\mathbf{S_{CO2}}$	$(\alpha_{C,alg} - (1 - f_{I,alg})Y_{alg,death}\alpha_{C,XS} - f_{I,alg}Y_{alg,death}\alpha_{C,XI})$	mg C L^{-1}
$\mathbf{S_{NH4}}$	$(\alpha_{N,alg} - (1 - f_{I,alg})Y_{alg,death}\alpha_{N,XS} - f_{I,alg}Y_{alg,death}\alpha_{N,XI})$	mg N L^{-1}
$\mathbf{S_H}$	$-\frac{1}{14}(\alpha_{N,alg} - (1 - f_{I,alg})Y_{alg,death}\alpha_{N,XS} - f_{I,alg}Y_{alg,death}\alpha_{N,XI})$	mmole H L^{-1}
$\mathbf{X_{alg}}$	-1	mg X_{alg} L^{-1}
$\mathbf{X_S}$	$(1 - f_{I,alg})Y_{alg,death}$	mg X_S L^{-1}
$\mathbf{X_I}$	$f_{I,alg}Y_{alg,death}$	mg X_I L^{-1}
4	***Heterotrophic growth of bacteria***	
$\mathbf{S_S}$	$-\frac{1}{Y_{bac,growth}}$	mg S_S L^{-1}
$\mathbf{S_{O2}}$	$\left(\frac{\alpha_{O,SS}}{Y_{bac,growth}} - \alpha_{O,bac}\right) - 8\left(\frac{\alpha_{H,SS}}{Y_{bac,growth}} - \alpha_{H,bac}\right) - \frac{8}{3}\left(\frac{\alpha_{C,SS}}{Y_{bac,growth}} - \alpha_{C,bac}\right) + \frac{12}{7}\left(\frac{\alpha_{N,SS}}{Y_{bac,growth}} - \alpha_{N,bac}\right)$	mg O_2 L^{-1}
$\mathbf{S_{CO2}}$	$\left(\frac{\alpha_{C,SS}}{Y_{bac,growth}} - \alpha_{C,bac}\right)$	mg C L^{-1}
$\mathbf{S_{NH4}}$	$\left(\frac{\alpha_{N,SS}}{Y_{bac,growth}} - \alpha_{N,bac}\right)$	mg N L^{-1}
$\mathbf{S_H}$	$-\frac{1}{14}\left(\frac{\alpha_{N,SS}}{Y_{bac,growth}} - \alpha_{N,bac}\right)$	mmole H L^{-1}
$\mathbf{X_{bac}}$	1	mg X_{bac} L^{-1}
5	***Aerobic endogenous respiration of bacteria***	
$\mathbf{S_{O2}}$	$-\frac{8}{3}(\alpha_{C,bac} - f_{I,bac}\alpha_{C,XI}) - 8(\alpha_{H,bac} - f_{I,bac}\alpha_{H,XI}) + (\alpha_{O,bac} - f_{I,bac}\alpha_{O,XI}) + \frac{12}{7}(\alpha_{N,bac} - f_{I,bac}\alpha_{N,XI})$	mg O_2 L^{-1}
$\mathbf{S_{CO2}}$	$\alpha_{C,bac} - f_{I,bac}\alpha_{C,XI}$	mg C L^{-1}

S_{NH4}	$\alpha_{N,bac} f_{I,bac} \alpha_{N,XI}$	mg N L^{-1}
S_H	$-\frac{1}{14}(\alpha_{H,bac} - f_{I,bac}\alpha_{H,XI})$	mmole H L^{-1}
X_{bac}	-1	mg X_{alg} L^{-1}
X_I	$f_{I,bac}$	mg X_I L^{-1}
6	***Death of bacteria***	
S_{O2}	$(\alpha_{O,bac} - (1 - f_{I,bac})Y_{bac,death}\alpha_{O,XS} - f_{I,bac}Y_{bac,death}\alpha_{O,XI}) - 8(\alpha_{H,bac} - (1 - f_{I,bac})Y_{bac,death}\alpha_{H,XS} - f_{I,bac}Y_{bac,death}\alpha_{H,XI}) - \frac{8}{3}(\alpha_{C,bac} - (1 - f_{I,bac})Y_{bac,death}\alpha_{C,XS} - f_{I,bac}Y_{bac,death}\alpha_{C,XI}) + \frac{12}{7}(\alpha_{N,bac} - (1 - f_{I,bac})Y_{bac,death}\alpha_{N,XS} - f_{I,bac}Y_{bac,death}\alpha_{N,XI})$	mg O_2 L^{-1}
S_{CO2}	$(\alpha_{C,bac} - (1 - f_{I,bac})Y_{bac,death}\alpha_{C,XS} - f_{I,bac}Y_{bac,death}\alpha_{C,XI})$	mg C L^{-1}
S_{NH4}	$(\alpha_{N,bac} - (1 - f_{I,bac})Y_{bac,death}\alpha_{N,XS} - f_{I,bac}Y_{bac,death}\alpha_{N,XI})$	mg N L^{-1}
S_H	$-\frac{1}{14}(\alpha_{N,bac} - (1 - f_{I,bac})Y_{bac,death}\alpha_{N,XS} - f_{I,bac}Y_{bac,death}\alpha_{N,XI})$	mmole H L^{-1}
X_{bac}	1	mg X_{bac} L^{-1}
X_S	$(1 - f_{I,bac})Y_{bac,death}$	mg X_S L^{-1}
X_I	$f_{I,bac}Y_{bac,death}$	mg X_I L^{-1}
7	***Hydrolysis***	
S_S	Y_{hyd}	mg S_S L^{-1}
S_{O2}	$(\alpha_{O,XS} - Y_{hyd}\alpha_{O,SS}) - 8(\alpha_{H,XS} - Y_{hyd}\alpha_{H,SS}) - \frac{8}{3}(\alpha_{C,XS} - Y_{hyd}\alpha_{C,SS}) + \frac{12}{7}(\alpha_{N,XS} - Y_{hyd}\alpha_{N,SS})$	mg O_2 L^{-1}
S_{CO2}	$(\alpha_{C,XS} - Y_{hyd}\alpha_{C,SS})$	mg C L^{-1}
S_{NH4}	$(\alpha_{N,XS} - Y_{hyd}\alpha_{N,SS})$	mg N L^{-1}
S_H	$-\frac{1}{14}(\alpha_{N,XS} - Y_{hyd}\alpha_{N,SS})$	mmole H L^{-1}
X_S	-1	mg X_S L^{-1}
8	***Equilibrium SCO2/SHCO3***	
S_{CO2}	-1	mg C L^{-1}
S_{HCO3}	1	mg C L^{-1}
S_H	$\frac{1}{12}$	mmole H L^{-1}
9	***Equilibrium SCO2/SHCO3***	
S_{HCO3}	-1	mg C L^{-1}
S_{CO3}	1	mg C L^{-1}
S_H	$\frac{1}{12}$	mmole H L^{-1}
10	***Equilibrium SH2O/(SH+SOH)***	
S_H	1	mmole H L^{-1}
S_{OH}	1	mmole OH L^{-1}
11	***Equilibrium SNH4/SNH3***	
S_{NH4}	-1	mg N L^{-1}
S_{NH3}	1	mg N L^{-1}
S_{OH}	$-\frac{1}{12}$	mmole OH L^{-1}
12	***Gas-liquid mass transfer of CO2***	

S_{CO2}	1	mg C L^{-1}
13	***Gas-liquid mass transfer of O2***	
S_{O2}	1	mg O_2 L^{-1}

Table 4: Kinetic parameters.

Symbol	Description	Value	Unit	Ref.
a	Absorption coefficient on a dry weight basis	0.2-0.3	m^2 g^{-1}	[b, c] (McKinney, 2004)
z	Total light path into reactor	0.03-0.5	m	[a]
I_{opt}	Optimized light incident for microalgae growth	45-90	µmol photons $m^{-2}s^{-1}$	[b, c] (Vonshak, 1997)
I_0	Light intensity at the surface of the reactor	Direct sunlight: ~1700 Fluorescent lamp: ~100-300	µmol photons $m^{-2}s^{-1}$	[a]
$\mu_{max,alg}$	Maximum specific growth rate of microalgae	1-4	d^{-1}	[b] (Packer et al., 2011; Reichert et al., 2001b)
$\mu_{max,bac}$	Maximum specific growth rate of bacteria	5-10	d^{-1}	[b]
b_{alg}	Decay rate coefficient of algae	0.1	d^{-1}	[b, c]
b_{bac}	Decay rate coefficient of bacteria	0.3-0.5	d^{-1}	[b]
$K_{NO3,alg}$	Half saturation constant for algae growth on NO_3^-	0.1	mg N L^{-1}	[b, c]
$K_{CO2,alg}$	Half saturation constant for algae growth on CO_2	0.5	mg C L^{-1}	[b, c]
$K_{O2,alg}$	Dissolved oxygen affinity constant for algae	0.2	mg O_2 L^{-1}	[b] (Reichert et al., 2001b)
$K_{SS,bac}$	Half-saturation coefficient of organic substances for bacteria	2	mg S_S L^{-1}	[a]
$K_{N,bac}$	Half-saturation coefficient of nitrogen substances for bacteria	0.2	mg N L^{-1}	[b]
$K_{O2,bac}$	Dissolved oxygen affinity constant for bacteria	0.2	mg O_2 L^{-1}	[b]
$k_{resp,alg}$	Endogenous respiration rate of algae	0.1	d^{-1}	[b, c]
$k_{resp,bac}$	Endogenous respiration rate of bacteria	0.2	d^{-1}	[b, c]
k_{hyd}	Hydrolysis rate	3	d^{-1}	[b, c]
K_{LaCO2}	Volumetric mass transfer coefficient of CO_2		d^{-1}	[a]
K_{LaO2}	Volumetric mass transfer coefficient of O_2	10-50	d^{-1}	[a]

[a] Parameters can be measured directly.
[b] Parameter values can be obtained from literatures.
[c] Parameter values can be obtained from literatures but without confidence, or needed to be estimated in different contents.

Appendix C

Enhanced lipid extraction from algae using free nitrous acid pretreatment

Bioresource Technology 159 (2014) 36–40

Contents lists available at ScienceDirect

Bioresource Technology

journal homepage: www.elsevier.com/locate/biortech

Enhanced lipid extraction from algae using free nitrous acid pretreatment

Xue Bai [a], Forough Ghasemi Naghdi [b], Liu Ye [a], Paul Lant [a], Steven Pratt [a,*]

[a] School of Chemical Engineering, The University of Queensland, Brisbane, QLD 4072, Australia
[b] School of Agriculture and Food Sciences, The University of Queensland, Brisbane, QLD 4072, Australia

HIGHLIGHTS

- Free nitrous acid (FNA) pretreatment enhances lipid extraction yield from algae.
- FNA pretreatment boosts mass transfer coefficient of lipid extraction significantly.
- FNA at ppm level can cause algal cell envelope (wall and membrane) disruption.
- Cell disruption is not accompanied by lipid release.
- Algal cell envelope disruption contributes to lipid extraction by organic solvent.

ARTICLE INFO

Article history:
Received 1 November 2013
Received in revised form 29 January 2014
Accepted 31 January 2014
Available online 8 February 2014

Keywords:
Free nitrous acid
Lipid extraction
Algae
Biodiesel
Cell disruption

ABSTRACT

Lipid extraction has been identified as a major bottleneck for large-scale algal biodiesel production. In this work free nitrous acid (FNA) is presented as an effective and low cost pretreatment to enhance lipid recovery from algae. Two batch tests, with a range of FNA additions, were conducted to disrupt algal cells prior to lipid extraction by organic solvents. Total accessible lipid content was quantified by the Bligh and Dyer method, and was found to increase with pretreatment time (up to 48 h) and FNA concentration (up to 2.19 mg HNO_2-N/L). Hexane extraction was used to study industrially accessible lipids. The mass transfer coefficient (k) for lipid extraction using hexane from algae treated with 2.19 mg HNO_2-N/L FNA was found to be dramatically higher than for extraction from untreated algae. Consistent with extraction results, cell disruption analysis indicated the disruption of the cell membrane barrier.

1. Introduction

Microalgae have many advantages compared to other energy crops, including a high growth rate, high biomass production, and minimal arable land use (Amin, 2009). In addition, many algal species can grow in wastewater, brackish water or seawater (Buchanan et al., 2012; Chaumont, 1993; Pittman et al., 2011), thereby avoiding requirement for fresh water, which is a limited resource in many parts of the world. Algal lipids, commonly referred to as crude lipids or total fatty acids of algae, are extracted and then converted by transesterification to algal biodiesel (Chisti, 2007). Considering the whole algal biodiesel production pipeline, lipid extraction, along with the dewatering of the algal biomass, is a relatively energy-intensive process and a major bottle-neck for large-scale production (Chisti, 2007; Halim et al., 2012). This is partly due to the fact that algae possess a cell envelope (cell wall and cell membrane), which is a thick and rigid layer consisting of complex carbohydrates and glycoproteins with high mechanical strength and chemical resistance (Kim et al., 2013).

Solvent extraction is one of the most widely applied methods to recover lipids. Traditional chloroform-based lipid extraction is effective for the lab-scale exercises, but an alternative organic solvent, like hexane, which is easier to handle and more cost effective, is needed for industrial scale activities (Halim et al., 2012). A challenge for solvent extraction methods is the physical algal cell envelope, which prevents direct contact between lipid and solvent. Mechanical, chemical and biological cell disruption techniques are widely studied as a means to overcome this problem (Lee et al., 2010; Prabakaran and Ravindran, 2011). Mechanical methods such as microwave pretreatment have been proven to be effective, but require high energy input. Alternative chemical and biological cell disruption techniques are attracting escalating attention (Jin et al., 2012; Lee et al., 2010; Mendes-Pinto et al.,

* Corresponding author. Tel.: +61 7 33467843.
E-mail address: s.pratt@uq.edu.au (S. Pratt).

http://dx.doi.org/10.1016/j.biortech.2014.01.133
0960-8524/

Abstract

Lipid extraction has been identified as a major bottleneck for large-scale algal biodiesel production. In this work free nitrous acid (FNA) is presented as an effective and low cost pretreatment to enhance lipid recovery from algae. Two batch tests, with a range of FNA additions, were conducted to disrupt algal cells prior to lipid extraction by organic solvents. Total accessible lipid content was quantified by the Bligh and Dyer method, and was found to increase with longer pretreatment time (48 h) and higher FNA concentration (up to 2.19 mg HNO_2-N/L). Hexane extraction was used to study industrially accessible lipids. The mass transfer coefficient (k) for lipid extraction using hexane from algae treated with 2.19 mg HNO_2-N/L FNA was found to increase dramatically. Consistent with extraction results, cell disruption analysis indicated the disruption of cell membrane barrier, thereby enhancing lipid extraction from algae.

Keywords: Free nitrous acid; Lipid extraction; Algae; Biodiesel; Cell disruption

1. Introduction

Microalgae have many advantages compared to other energy crops, including a high growth rate, high biomass production, and minimal arable land use (Amin, 2009). In addition, many algal species can grow in waste water, brackish water or seawater (Buchanan et al., 2012; Chaumont, 1993; Pittman et al., 2011), thereby avoiding requirement for fresh water, which is a limited resource in many parts of the world. Algal lipids, commonly referred to as crude lipids or total fatty acids of algae, are extracted and then convereted by transesterification to algal biodiesel (Chisti, 2007). Considering the whole algal biodiesel production pipeline, lipid extraction, along with the dewatering of the algal biomass, is a relatively energy-intensive process and a major bottle-neck for large-scale production (Chisti, 2007; Halim et al., 2012). The main reason for high energy consumption for lipid extraction is that algae possess a cell envelope (cell wall and cell membrane), which is a thick and rigid layer consisting of complex carbohydrates and glycoproteins with high mechanical strength and chemical resistance (Kim et al., 2013).

Solvent extraction is one of the most widely applied methods to recover lipids, but a great challenge of solvent extraction of lipids from algae is the low extraction yield from wet algal biomass. Even though the traditional chloroform-based lipid extraction protocol is effective for the lab-scale analyses,

an alternative organic solvent, hexane which is more user-friendly and cost effective, is needed for scale-up (Halim et al., 2012). When solvent-based extraction techniques are applied to a wet algal sample, cells tend to remain in the aqueous phase due to their cell envelops, which prevent them from getting direct contact with solvent. Mechanical, chemical and biological cell disruption techniques are widely studied ((Lee et al., 2010; Prabakaran and Ravindran, 2011). Mechanical methods such as microwave show ability to disrupt cells effectively however high energy cost prevented the large scale application. Chemical or biological cell disruption techniques are attracting escalating attention to improve lipid extraction yield or efficiency (Jin et al., 2012; Lee et al., 2010; Mendes-Pinto et al., 2001). Despite the high cell-disruption performance of the chemical and biological treatments, the potential to scale up these methods is still limited since they require continuous use of expensive chemicals and enzymes (Jin et al., 2012; Mendes-Pinto et al., 2001).

Recent studies showed that FNA, which is the protonated form of nitrite, has strong cellular destruction and enzyme interference effects on several microorganisms (Jiang et al., 2011). FNA has been regarded as a biochemical reagent since several works also revealed that FNA and its derivatives such as nitric oxide radical ($NO\cdot$) and nitrous anhydride (N_2O_3) have effect on protein and polysaccharides degradation (Dedon and Tannenbaum, 2004). Hence FNA has been applied in the water and wastewater industry for sludge treatment, biofilm control etc. However, no study has been reported so far on the effect of FNA pretreatment on algal cells. Since FNA combines chemical effect and biological effect on algal cells, our hypothesis is that FNA pretreatment could help disrupt the algal cell envelope barrier, thereby increasing the rate of lipid mass transfer from the algal cells into the organic solvent.

The aim of this study is to assess a novel FNA pretreatment method to increase lipid extraction yield and extraction efficiency from algal biomass. Moreover, FNA is a green and renewable chemical that can be produced by nitritation from anaerobic digester liquor (Wang et al., 2013). In this study, algal cells were disrupted using different concentrations of FNA prior to lipid extraction by organic solvents. A traditional lab-scale lipid extraction method, Bligh and Dyer method (Bligh and Dyer, 1959), was applied to assess the lipid extraction yields from disrupted cells. Furthermore, hexane extraction was applied to examine the lipid extraction efficiency by modelling and studying the extraction kinetics. We also evaluated the effect of FNA on cell disruption by fluorescence staining, Scanning Electron Microscopy (SEM) and intracellular compounds release.

2. Materials and Methods

2.1. Microalgae cultivation and harvest

Tetraselmis striata M8, which is a marine algae with high lipid accumulation ability, was cultured in 40 L airlift open photobioreactors at the University of Queensland. F/2 medium was used as the growth media. Culture pH was kept constant at 8.5±0.2 by CO_2 injection with an electronic controller, and the depletion of nutrients ($NO_3^-$and PO_4^{3-}) was tested using seawater aquaria nutrients kits (DAPI Aquarium Pharmaceuticals for NO_3^- and Nutrafin for PO_4^{3-}). Nitrogen starvation strategy was used to accumulate lipids in the algal cells. After 5 days cultivation for algae growth and 3 days for lipid accumulation, 90 L algal culture was concentrated to paste by centrifugation in a Beckman Coulter, Allegra™ X-12 at 3750 g for 3 min in 800 ml batches. Centrifugation was conducted for a short time to avoid mechanical cell disruption.

2.2. FNA pretreatment

The work plan comprised two batches of four experiments. The experimental conditions are summarized in Table 1. Algal culture and lipid accumulation was as described in Section 2.1. The collected algal pellet was re-suspended with de-ionized water and then the mixed liquor was evenly distributed between four beakers (reactor volume 500 ml) for FNA pretreatment.

Predetermined amounts of sodium nitrite stock solution (30 g N /L) were added to the batch reactors in different volumes at the beginning of each experiment, which resulted in initial concentrations of nitrite varying between 0 and 1200 mg NO_2-N/L, as summarized in Table 1. Batch 1 was treated for 48 h and batch 2 was treated for 60 h. pH was kept approximately constant during the whole pretreatment at 5.0±0.2 or 6.0±0.2 (according to Table 1) through manually adding 0.5 M HCl. All reactors were well mixed by magnetic stirring at constant speed of 350 rpm. The experimental procedure is presented in Table 1. The concentration of FNA was varied by controlling the nitrite concentration under pH 5 or 6 as described. The FNA concentration was calculated using the following formula.

$$\mathrm{FNA(mg\ HNO_2\text{-}N/L)} = S_{NO2^- - N}/(K_a \times 10^{pH}) \quad (1)$$

where $S_{NO2^- - N}$ as dissolved NO_2^- concentration (mg N/L) and $K_a = e^{-2{,}300/(273.15+T)}$ at an operation temperature T (ºC) (25ºC in this study) (Anthonisen et al., 1976).

In each batch test, mixed liquor samples were taken approximately every 12 hours using a syringe and immediately filtered through disposable Millipore filter units (0.22 μm pore size) for the off-line analysis.

Standard microwave was applied as a control to untreated algal biomass using a microwave oven at a high temperature (about 100ºC and 2450 MHz) for 5 min.

2.3. Evaluation of cell disruption

2.3.1 Cell membrane damage assay

Microscope observation and cell membrane damage assays were performed before and after pretreatment. Untreated algae and algae samples from each reactor after 48 h pretreatment were diluted 10 times before staining. The SYTOX Green fluorescent probe (Invitrogen, Ltd., UK) was supplied as a 5 mM stock solution in DMSO. 0.5 μl of this stock solution was added to 0.5 ml cell suspension giving a final dye concentration of 5 μM, and the mixture was incubated for 5 minutes at room temperature in the dark. Algal cells in 24 random fields were counted under a fluorescence microscope (BX61, Olympus, Tokyo, Japan) equipped with a double band pass filter set at 473-498 and 548-573 nm for excitation, and at 515-535 and 590-620 nm for emission. Damaged cell to intact cell ratio was then calculated. Images were obtained and post-adjusted with DPC controller 1.2.1 (Olympus optical co. LTD.) and iTEM 5.0 (Olympus Soft Imagining Solutions, Olympus optical co. LTD.) respectively.

2.3.2 Scanning electron microscopy (SEM) observation

SEM analysis was performed as described by Naveena *et al.* (Naveena et al., 2005). Briefly, cells were fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2), dehydrated in ethanol (70%, 90% and absolute), and dried with liquid CO_2. The dried samples were coated with layers of platinum using a SPI module sputter coater. A Phillips XL-30 SEM was used to observe the surface morphology of the algal cells. The SEM images were taken at an acceleration voltage of 10 kV.

2.3.3 Physicochemical characterization

Total suspended solids (TSS), volatile suspended solids (VSS), total chemical oxygen demand (TCOD) and soluble chemical oxygen demand (SCOD) were measured according to standard methods (American Public Health Association, 2005). TCOD and SCOD were measured using

Spectroquant® photometric cell tests (114541 and 114555, Merk, Germany), a Thermoreactor TR 300 (Merck, Germany) and a UV-Visible Spectrophotometer (Varian Cary®50, Varian, Inc., Australia). NH_4^+-N, NO_3^--N, NO_2^--N, PO_4^{3-}-P, total Kjeldahl phosphorous (TKP), total Kjeldahl nitrogen (TKN) and total soluble Kjeldahl nitrogen (STKN) were measured with a Lachat QuikChem 8000 Flow Injection Analyser (Lachat Instrument, Milwau-kee, USA). The protein concentration was measured by the BCA method with BSA as standard (Smith et al., 1985). The polysaccharide concentration was determined by the Anthrone method with glucose as standard (Raunkjær et al., 1994). Lipid content of supernatant was measured by an InfraCal TOG/TPH Analyzer (Wilks Enterprise, Inc., USA).

2.4. Lipid extractions

The algal liquor samples were taken from pre-treatment tests Batch 1 and 2 in duplicate. After the samples were taken, each sample was centrifuged at 3270 g (Beckman Coulter, Allegra™ X-12) for 3 min and the pellet was collected for lipid extraction analysis (in duplicate). Samples were taken from batch 1 (Exp.1-4) through the pretreatment at 5 h, 24 h and 48 h and samples were taken from batch 2 (Exp. 5-8) at the end of pretreatment (60 h).

The total lipids were extracted by chloroform : methanol (1:1, v/v) with the collected wet algal biomass using a slightly modified version of Bligh and Dyer method (1959). 3 g of wet algal paste was treated with 20 ml of solvent, followed by adding 10 ml de-ionised water. The bottom chloroform layer of the extracts was dried in vacuum until constant weight. Moisture of algal pastes was determined by weighing certain amount of wet paste on filter paper (GF/C, Whatman) and dried in 105 °C oven overnight, all in triplicates. Lipid extraction from untreated and microwaved samples was operated exactly as given in the previous paragraph.

2.5. Kinetic analysis of lipid extraction

The algal liquor from the end of pretreatment tests of batch 1 was centrifuged at 3270 g (Beckman Coulter, Allegra™ X-12) for 3 min. Lipid extraction kinetics of the treated algal biomass was studied by serial Soxhlet extraction apparatus (Wang and Weller, 2006) using n-hexane : ethanol (3:1, v/v) as extraction solvent. Lipid extraction yields were quantified for 2 h and 6 h extraction. The Soxhlet extractions were done in duplicate for each sample.

Parameters describing the lipid extraction process were determined by fitting the following first-order kinetic model to the experimental data using SigmaPlot® software (version 12; Systat Software, San

Jose, CA, USA). This model was widely applied for the analysis of dissolution and leaching within liquid-solid systems (Fajardo et al., 2007; Halim et al., 2011):

$$Y(t) = Y_o(1 - e^{-kt}) \qquad (2)$$

Where $Y(t)$ (wt% of dried algal biomass) is the lipid yield extracted by the organic solvent at time t, Y_o (wt% of dried algal biomass) is the amount of lipid originally present in the microalgal cells, k (h^{-1}) is the lipid mass transfer coefficient from the algal cells into the organic solvent, t (h) is the extraction time.

2.6. Statistical analysis

All analyses were performed in duplicate. All mean values were analysed by one-way ANOVA and Tukey test (SigmaPlot® software) at $p < 0.05$ to detect significant differences.

Table 1: Experimental conditions applied in batch tests and outputs.

		Batch1				**Batch 2**			
		Exp. 1	**Exp. 2**	**Exp. 3**	**Exp. 4**	**Exp. 5**	**Exp. 6**	**Exp. 7**	**Exp. 8**
Conditions	pH	6	6	6	6	5	5	5	5
	NO_2^- (mg N/L)	0	100	450	900	0	100	600	1200
	FNA (mg HNO_2-N/L)	0	0.24	1.09	2.19	0	2.25	13.49	26.98
Appearance & cell damage	Culture appearance	Dark green	Dark green	Yellowish -green	Yellowish -green	Dark green	Yellowish -green	Brown	Brown
	membrane damaged cell increase (%)[c]	69	72	80	92	85	96	96	96
Analysis external to cells and cell debris	SCOD increase (% released SCOD/TCOD)[a]	ND	7.91	10.53	11.48	ND	15.62	15.57	15.33
	Lipid (mg /L)	ND	ND	ND	ND	ND	ND	ND	ND
	Protein (mg /L)	57.38	212.42	245.42	338.50	117.42	832.08	929.67	983.17
	Polysaccharides (mg /L)	52.09	107.76	186.29	235.32	47.84	144.07	272.39	529.92
	NH_4^+ increase (mg N/L)[b]	6.77	4.85	3.59	3.18	30.40	9.32	8.12	6.10

[a, b] SCOD and NH_4^+ was measured after filtering by 0.22 µm membrane filter.
[c] Damaged cell to intact cell ratio was quantified using SYTOX Green fluorescence staining. ND: not detected. All outputs data is after 48 h pretreatment.

3. Results and Discussion

3.1. Effect of FNA on algal cell disruption

Table 1 compares the pretreatment results of eight experiments. When the FNA concentration reached 1.09 mg HNO_2-N/L (Exp. 3, 4, and 6), the colour of the algal liquor turned to yellowish-green from dark green (natural colour). With a further increase in FNA concentration to 26.98 mg HNO_2-N/L (Exp. 7 and 8), the colour turned to brown, indicating denaturation of chlorophyll pigments in the algal cells. This result is consistent with the microscope observations (Fig. S1) Under the fluorescent microscope, chlorophyll fluoresced as red colour. But seldom red autofluorescence was observed in Exp. 7 and Exp. 8 (Fig. S1g) after 48 h pretreatment, although the cells retained autofluorescence in Exp. 6. The reduction in autofluorescence matches the change of liquor colour from yellowish-green to brown after extremely high FNA concentration pretreatment, confirming the denaturation of chlorophyll pigments. Severe treatment conditions Exp. 7 and Exp. 8 even caused some cells to disintegrate into fragments (Fig. S1f, yellow circle).

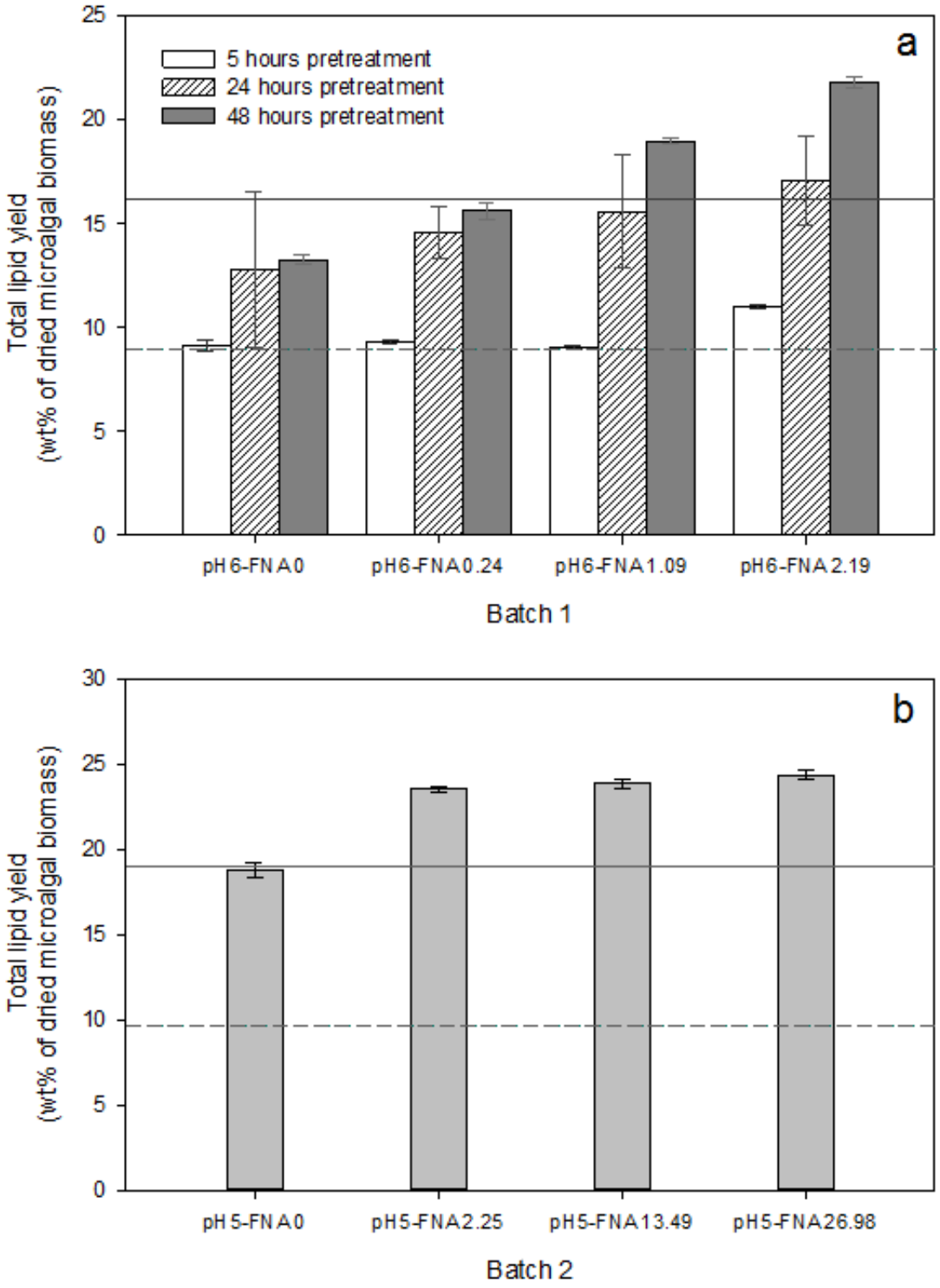


Fig. 1: Results of lipid yields by Bligh and Dyer extraction in different experiments. Error bars indicate standard error in duplicates tests: (a) results from batch 1; (b) results from batch 2. Dashed lines show lipid yields from untreated algal biomass; solid lines show lipid yields from microwaved algal biomass. Error bars are standard errors, n=2.

After 48 h pretreatment, the release of soluble components (being standardized as SCOD/TCOD ratio increase compared to control experiments) increased from 7.9% to 15.6 %. The result indicates that some soluble intracellular components were released into the liquor, and some large molecules or cell structures such as flagella were severely disrupted to small molecules, smaller than the pore size of filter membrane (0.22 μm). This result is generally higher than the SCOD release reported by Sheng *et al.* (Sheng et al., 2011), who applied pulsed-electric-field pretreatment to cyanobacteria, indicating that FNA pretreatment is a relatively severe pretreatment technology.

The characteristics of the material released into solution are presented in Table 1. The data shows that the material mainly consisted of protein and polysaccharides; no lipids were detected in solution. The absence of external lipid was verified by Nile Red staining (see Fig. S2).

Disruption of the algal cell membrane was visualized and analysed by counting 24 random views under fluorescent microscope after SYTOX Green staining. SYTOX Green has a high affinity to nucleic acid in the cells and can emit bright green fluorescence light when excited with a 450-490nm source (Sato et al., 2004). Since it is a large molecule, SYTOX Green could only penetrate into membrane damaged cells and autofluorescence of chlorophyll were observed simultaneously, which allows the discrimination of membrane damaged cells and intact cells. The linear correlation for M8 species between the real percentage of live algae in the samples and that measured by this method was tested prior to this study (data not shown here). Membrane damaged cell ratio increase was observed in all the systems over the 48 h pretreatment (Table 1). At pH 6, increasing FNA concentration resulted in an increase in damaged cell ratio (from 69% to 92%), which indicates a strong correlation of the biocidal effect with FNA concentration. Similar trends of biocidal effect toward microorganisms residing in sewer biofilms has been reported (Jiang et al., 2011). As FNA concentration increased (Exp. 6-8), the damage to the cell membrane also became more severe. Meanwhile, comparing Exp. 1 and Exp. 5 with dropping pH from 6 to 5, damaged cell ratio increased to from 69% to 85%, which indicates an effect of pH decrease to membrane damage. It is worth noting that, although cell membrane was disrupted by the pretreatment, lipid remained in the algal cells. This was verified by checking fluorescence of treated liquor after Nile Red staining (see Fig. S2).

Cell samples of untreated algal culture and Exp. 4 algal cells after 48 h pretreatment were analysed using SEM. Algal cells without treatment appeared to have smooth and tight surfaces (Fig. S1h), however when cells were treated with 2.19 mg HNO2-N/L FNA, cell surfaces shrank and some cells

collapsed. The process of FNA pretreatment certainly led to a surface defection, but again, despite disruption of the cell membranes, lipid remained in the algae (Table 1).

3.2. Lipid extraction

Algal biomass samples were taken from batch 1 through the pretreatment at 5 h, 24 h and 48 h, to evaluate the effect of different FNA concentrations on lipid extraction efficiency as a function of pretreatment time. Samples were taken from batch 2 at the end of pretreatment (60 h) to evaluate the contribution of higher FNA concentrations and longer pretreatment time. In line with our hypothesis, due to the cell disruption effects by FNA pretreatment, lipid extraction yield was boosted dramatically for all experiments compared to untreated algal biomass, confirming that the intracellular lipids became more available for solvent extraction after FNA pretreatment. Fig. 1 shows the results of lipid extraction yields by using Bligh and Dyer method from batch 1 (Fig. 1a) and batch 2 (Fig. 1b). After 5 h pretreatment, lipid yields of Exp. 1-3 were similar to that of untreated algal biomass (dashed line), whereas Exp. 4 shows a 22% increase comparing to untreated algal biomass. 24 h pretreatment by different concentrations of FNA enhanced lipid yields dramatically comparing to that from untreated algal biomass. The lipid yields of Exp. 4 after 24 h pretreatment generated an 89% increase to untreated algal biomass, to as high as 17.0±1.5% (wt% of dried algal biomass). This result is even 4% higher than the yield from algal biomass by standard microwave pretreatment which has been reported as one of the most efficient pretreatment methods for the tested green algae, vegetables and animal feeds (Lee et al., 2010; Mahesar et al., 2008 ; Virot et al., 2008). With longer pretreatment, lipid yields after 48 h pretreatment kept increasing; meanwhile the degree of increase was heightened by increasing FNA concentration. For Exp. 3 and 4, yielded lipid content reached 19.0±0.1% and 21.9±0.2% (wt% of dried algal biomass) respectively, a 2.1-fold and 2.4-fold increase over the untreated algae, which confirms the great potential of using FNA as novel pretreatment technique for lipid extraction from algal biomass.

An extended pretreatment (60h) was trialled for batch 2. Results (Fig. 1b) show that different concentrations of FNA, higher than 2.25 mg HNO_2-N/L up to 26.98 mg HNO_2-N /L, all had a similar effect on lipid extraction, which boosted the lipid extraction yield 2.5-fold in comparison with that from untreated algal biomass (dashed line). The result of batch 2 suggests that continuing to increase FNA concentration and pretreatment time does not significantly improve lipid extraction; 24.4±0.3% (wt% of dried algal biomass) is approaching the lipid content of algal biomass of batch 2. It is worth noting that, there is a challenge in optimising pretreatment time with extended times resulting in

improved yields but potentially limiting industrial throughput.

3.3. Lipid extraction kinetics

Although the Bligh and Dyer method has been widely applied for the majority of algal lipid analyses in the lab, the application of n-hexane, with lower toxicity and more potential for industrial scale-up, is needed to be studied. To evaluate the performance of n-hexane extraction, and to further evaluate the lipid extraction speed after different concentrations of FNA pretreatment, dynamic hexane lipid extraction by Soxhlet apparatus was operated and lipid yields were gravimetrically quantified after 2 h and 6 h. The lipid extraction results, with best-fit of the first-order kinetic models are shown in Fig. 2. The amount of lipid originally present in the algal biomass Y_o (wt% of dried algal biomass) was fixed as 21.75% (wt% of dried algal biomass), which was the maximum lipid content according to the Bligh and Dyer method. Table 2 summarises extraction parameter k (h^{-1}), which is the lipid mass transfer coefficient (from the algal cells into the organic solvent), and the R-square of regressions. With increased FNA concentration from 0 to 2.9 mg HNO2-N/L, the mass transfer coefficient k increased from 0.39 h^{-1} to 0.96 h^{-1}. This 2.5-fold increase supports the conclusion that the intracellular lipids became more readily available for solvent extraction after FNA pretreatment. Experimental results and microscope observation revealed that one of the main reasons for the increased lipid extraction yields by FNA pretreatment was the degradation of cell membrane and cell walls, which was achieved without associated lipid release (Table 1and Fig. S2).

Research on reactive nitrogen species have shown that FNA and its derivatives such as nitric oxide ($NO\cdot$) and nitrous anhydride (N_2O_3) have effect on protein and polysaccharides degradation (Dedon and Tannenbaum, 2004), suggesting it can disrupt the barrier to solvent penetration (cell membrane and cell wall) to help non-polar solvents like n-hexane more easily get access to the intracellular lipids. Since lipid extraction is largely limited by the contact time of solvent with intracellular lipids, disrupting the solvent barrier can increase the lipid extraction rate thereby shortening the extraction time.

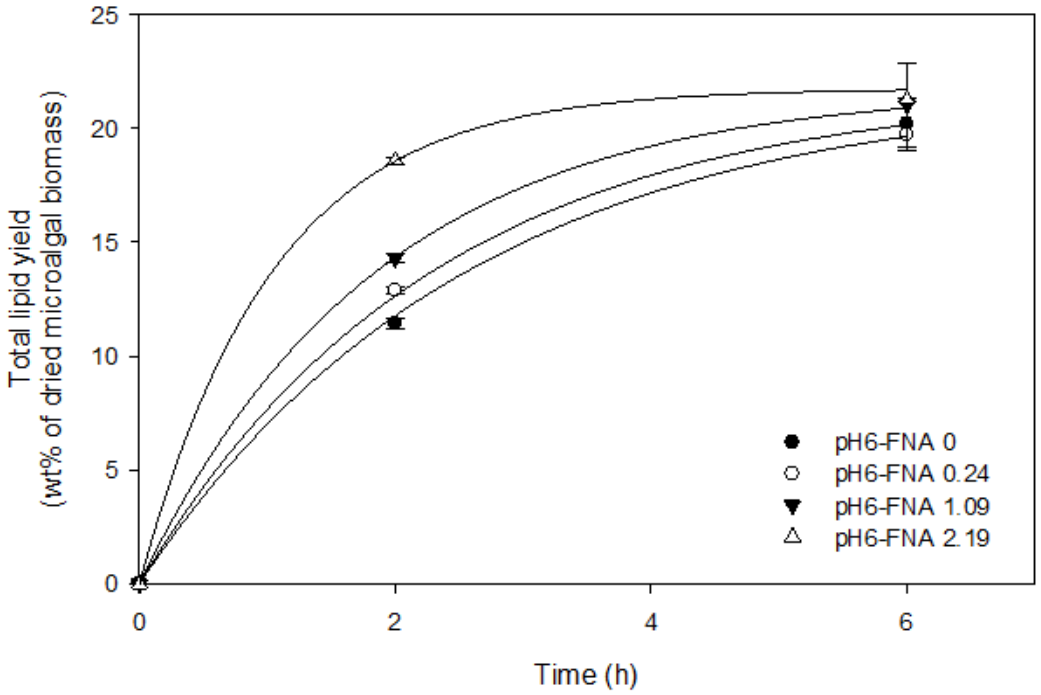


Fig. 2: Results of lipid yields by Soxhlet extraction. Lines show the first order kinetic fitting curves. Error bars are standard errors, n=2.

Table 2: Lipid Soxhlet extraction kinetics parameters and solvent recovery rate (mean ± standard error).

Exp.	k (h^{-1})	R^2	r_2 (% solvent recovered after 2 h extraction)	r_6 (% solvent recovered after 6 h extraction)
pH6-FNA 0	0.39±0.02	0.9980	69.2±0.0	61.5±0.0
pH6-FNA 0.24	0.43±0.02	0.9988	72.1±0.0	63.5±0.0
pH6-FNA 1.09	0.54±0.01	0.9999	73.1±0.0	65.4±0.0
pH6-FNA 2.19	0.96±0.05	0.9993	76.0±0.0	67.3±0.1

4. Conclusions

The total lipid extraction yield from algae was found to be enhanced with higher FNA concentration (up to 2.19 mg HNO_2-N/L) and longer pretreatment time (48 h). The highest total lipid extraction yield was found to be 21.9±0.2% (wt% of dried algal biomass), which was 2.4-fold higher compared to that from untreated algae. Also, the mass transfer coefficient (k) for lipid extraction using hexane from algae treated with 2.19 mg HNO_2-N/L FNA was found to increase dramatically. Therefore, it was concluded that the FNA pretreatment technique is a promising method to enhance lipid extraction from algal biomass.

Acknowledgements

The authors wish to thank Prof. Zhiguo Yuan for his promotion of FNA technology for cell disruption; Prof. Peer Schenk and Mr. Rakesh Narala of the Algae Biotechnology Group (The University of Queensland, Australia), for providing the algal inoculum and culturing protocols; Ms Robyn Webb of the Centre for Microscopy and Microanalysis (The University of Queensland, Australia) for her help on preparing SEM sample, and Mr. Masuduz Zaman for his help on SEM imaging.

Reference

American Public Health Association. 2005. Standard methods for the examination of water and wastewater. APHA-AWWA-WEF, Washington, DC.

Amin, S. 2009. Review on biofuel oil and gas production processes from microalgae. Energ. Convers. Manage, 50, 1834-1840.

Anthonisen, A.C., Loehr, R.C., Prakasam, T.B.S., Srinath, E.G. 1976. Inhibition of nitrification by ammonia and nitrous acid. Journal (Water Pollution Control Federation), 48, 835-852.

Bligh, E., Dyer, W. 1959. A rapid method of total lipid extraction and purification. Can. J. Biochem. Physiol, 37, 911-917.

Buchanan, J.J., Slater, F.R., Bai, X., Pratt, S. 2012. Algal growth and community structure in a mixed-culture system using coal seam gas water as the water source. Environ. Technol., 34, 695-701.

Chaumont, D. 1993. Biotechnology of algal biomass production: a review of systems for outdoor mass culture. J. Appl. Phycol., 5, 593-604.

Chisti, Y. 2007. Biodiesel from microalgae. Biotechnol. Adv., 25, 294-306.

Dedon, P.C., Tannenbaum, S.R. 2004. Reactive nitrogen species in the chemical biology of inflammation. Arch. Biochem. Biophys., 423, 12-22.

Fajardo, A.R., Cerdán, L.E., Medina, A.R., Fernández, F.G.A., Moreno, P.A.G., Grima, E.M. 2007. Lipid extraction from the microalga Phaeodactylum tricornutum. Eur. J. Lipid. Sci. Tech, 109, 120-126.

Halim, R., Danquah, M.K., Webley, P.A. 2012. Extraction of oil from microalgae for biodiesel production: A review. Biotechnol. Adv., 30, 709-732.

Halim, R., Gladman, B., Danquah, M.K., Webley, P.A. 2011. Oil extraction from microalgae for biodiesel production. Bioresour. Technol., 102, 178-185.

Jiang, G., Gutierrez, O., Yuan, Z. 2011. The strong biocidal effect of free nitrous acid on anaerobic sewer biofilms. Water Res., 45, 3735-43.

Jin, G., Yang, F., Hu, C., Shen, H., Zhao, Z.K. 2012. Enzyme-assisted extraction of lipids directly from the culture of the oleaginous yeast Rhodosporidium toruloides. Bioresour. Technol., 111, 378-382.

Kim, J., Yoo, G., Lee, H., Lim, J., Kim, K., Kim, C.W., Park, M.S., Yang, J.-W. 2013. Methods of downstream processing for the production of biodiesel from microalgae. Biotechnol. Adv., 31, 862–876.

Lee, J.-Y., Yoo, C., Jun, S.-Y., Ahn, C.-Y., Oh, H.-M. 2010. Comparison of several methods for effective lipid extraction from microalgae. Bioresour. Technol., 101, S75-S77.

Mahesar, S., Sherazi, S., Abro, K., Kandhro, A., Bhanger, M., Van de Voort, F., Sedman, J. 2008. Application of microwave heating for the fast extraction of fat content from the poultry feeds. Talanta, 75, 1240-1244.

Mendes-Pinto, M.M., Raposo, M.F.J., Bowen, J., Young, A.J., Morais, R. 2001. Evaluation of different cell disruption processes on encysted cells of Haematococcus pluvialis: effects on astaxanthin recovery and implications for bio-availability. J. Appl. Phycol., 13, 19-24.

Naveena, B.J., Altaf, M., Bhadrayya, K., Madhavendra, S.S., Reddy, G. 2005. Direct fermentation of starch to L(+) lactic acid in SSF by Lactobacillus amylophilus GV6 using wheat bran as support and substrate: medium optimization using RSM. Process. Biochem., 40, 681-690.

Prabakaran, P., Ravindran, A.D. 2011. A comparative study on effective cell disruption methods for lipid extraction from microalgae. Lett. Appl. Microbiol., 53, 150-154.

Pittman, J.K., Dean, A.P., Osundeko, O. 2011. The potential of sustainable algal biofuel production using wastewater resources. Bioresour. Technol., 102, 17-25.

Raunkjær, K., Hvitved-Jacobsen, T., Nielsen, P.H. 1994. Measurement of pools of protein, carbohydrate and lipid in domestic wastewater. Water Res., 28, 251-262.

Sato, M., Murata, Y., Mizusawa, M., Iwahashi, H., Oka, S.-i. 2004. A simple and rapid dual-fluorescence viability assay for microalgae. Microbiol Cult Coll, 20, 53-59.

Sheng, J., Vannela, R., Rittmann, B.E. 2011. Evaluation of cell-disruption effects of pulsed-electric-field treatment of Synechocystis PCC 6803. Environ. Sci. Technol., 45, 3795-3802.

Smith, P.K., Krohn, R.I., Hermanson, G.T., Mallia, A.K., Gartner, F.H., Provenzano, M.D., Fujimoto, E.K., Goeke, N.M., Olson, B.J., Klenk, D.C. 1985. Measurement of protein using bicinchoninic acid. Anal. Biochem., 150, 76-85.

Virot, M., Tomao, V., Ginies, C., Visinoni, F., Chemat, F. 2008. Microwave-integrated extraction of total fats and oils. J. Chromatogr. A, 1196–1197, 57-64.

Wang, L., Weller, C.L. 2006. Recent advances in extraction of nutraceuticals from plants. Trends Food Sci. Tech, 17, 300-312.

Wang, Q., Ye, L., Jiang, G., Jensen, P.D., Batstone, D.J., Yuan, Z. 2013. Free Nitrous Acid (FNA)-Based Pretreatment Enhances Methane Production from Waste Activated Sludge. Environ. Sci. Technol., 47, 11897-11904.

Supporting information

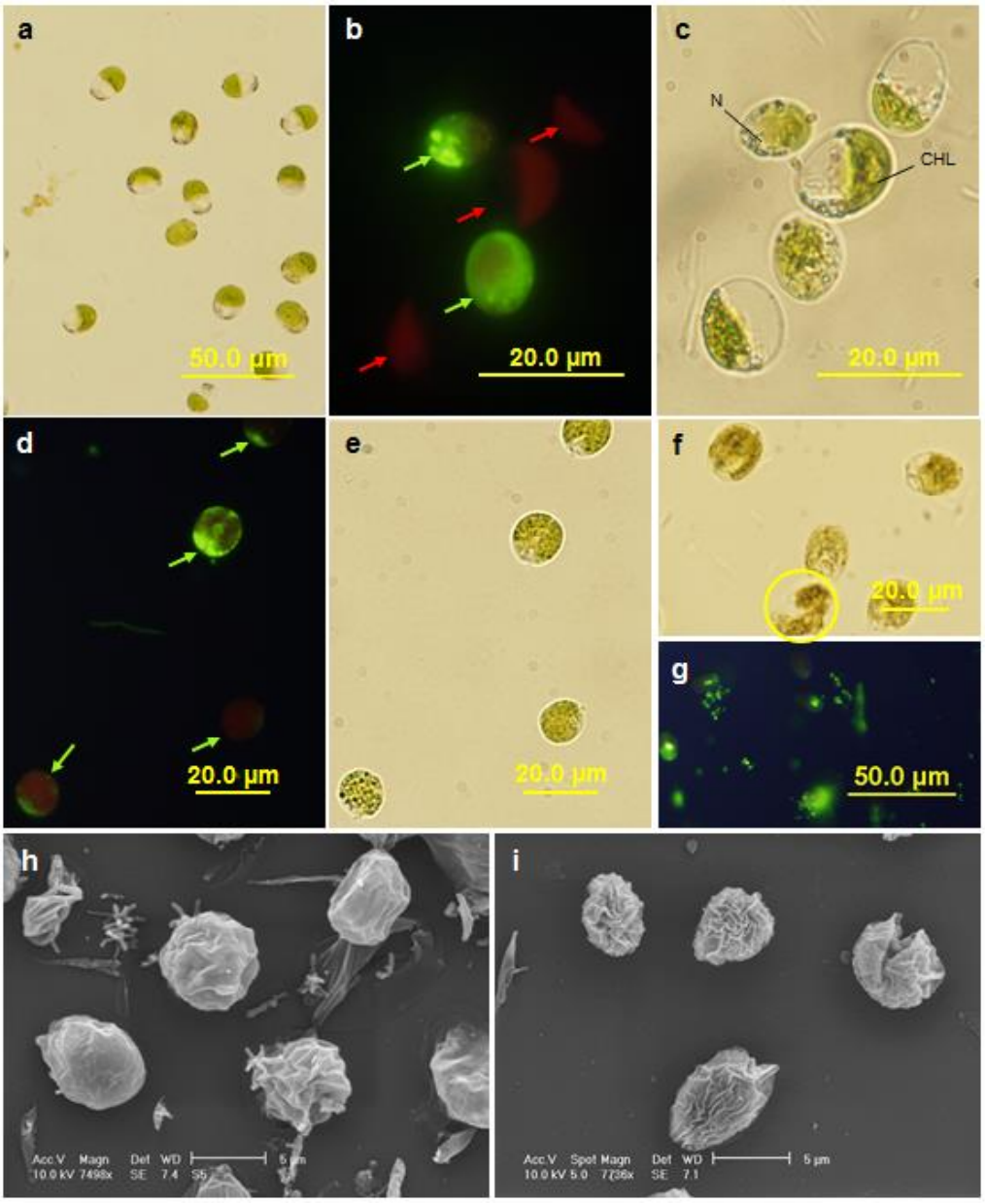


Fig. S1: Light microscope and SEM images of untreated and FNA-treated cells under different conditions. (a) untreated cells. (b) Exp. 1 (pH6-FNA 0) algal cells after 48 h pretreatment. (c) fluorescent image of the same field of image b. (d) Exp. 4 (pH6-FNA 2.19) algal cells after 48 h pretreatment. (e) fluorescent image of the same field of image d. (f) Exp. 8 (pH5-FNA 26.98) algal cells after 48 h pretreatment. (g) fluorescent image of Exp. 8. (h) and (i) SEM images of untreated cells and Exp. 4 algal cells after 48 h pretreatment. Green arrows show membrane damaged cells; Red arrows show intact cells. Yellow circle shows cell fragmentation. N: Nucleus. CHL: Chloroplast.

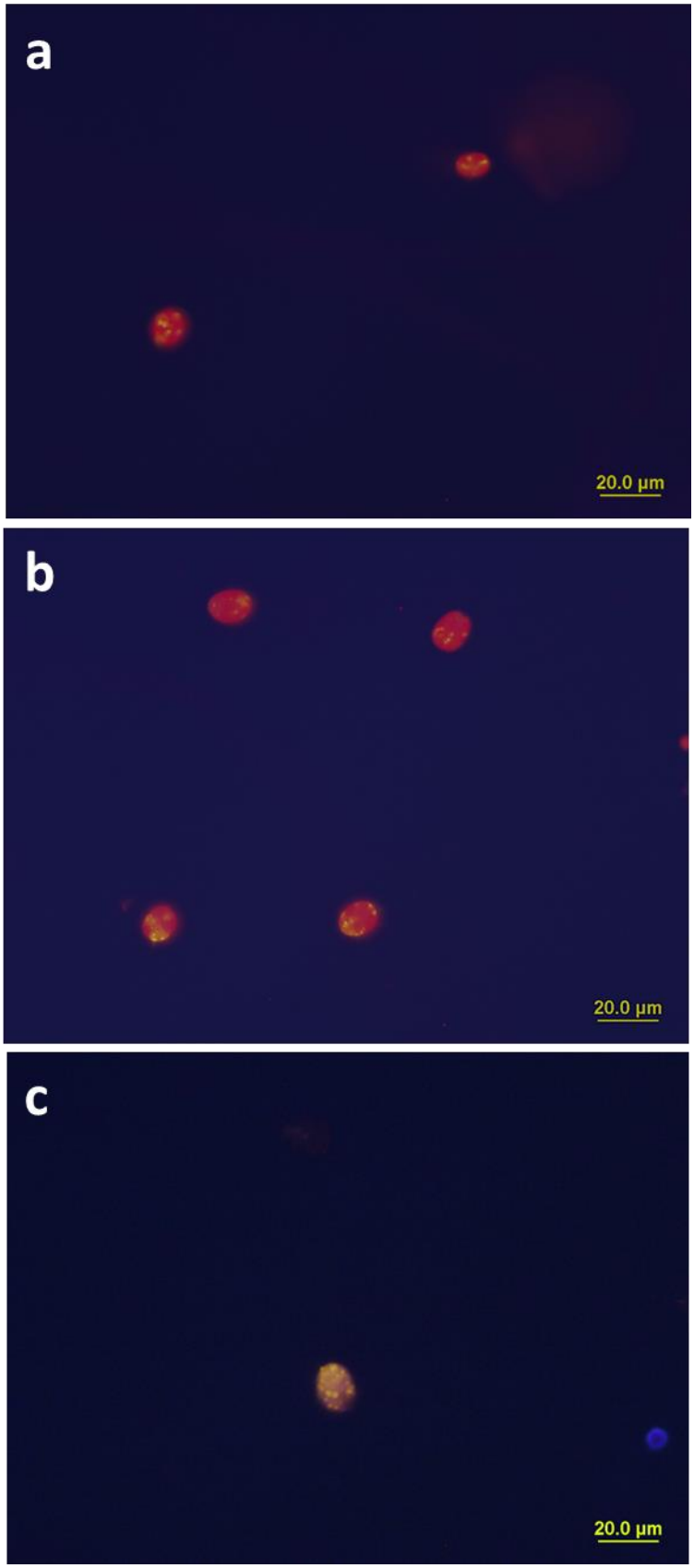


Fig. S2: Nile Red staining light microscope images of untreated and FNA-treated cells under different conditions. (a) untreated cells. (b) Exp. 4 (pH6-FNA 2.19) algal cells after 48 h pre-treatment. (c) Exp. 8 (pH5-FNA 26.98) algal cells after 48 h pretreatment.

Appendix D

Enhanced triacylglyceride (TAG) extraction from algae using free nitrous acid pre-treatment

Elsevier Editorial System(tm) for Applied Energy
Manuscript Draft

Manuscript Number: APEN-D-14-04471

Title: Enhanced triacylglyceride extraction from microalgae using free nitrous acid pre-treatment

Article Type: Original Paper

Keywords: biodiesel; fatty acid; free nitrous acid; microalgae; triacylglyceride

Corresponding Author: Dr. Steven Pratt, Ph.D.

Corresponding Author's Institution: University of Queensland

First Author: Xue Bai

Order of Authors: Xue Bai; Peer M Schenk; Zhiguo Yuan; Paul A Lant; Steven Pratt

Abstract: Triacylglyceride (TAG) recovery from algal biomass is primarily limited by the rigid algal cell envelope (cell wall and cell membrane). In this work, the effect of free nitrous acid (FNA) pre-treatment on TAG recovery from algal biomass with six different FNA concentrations is reported. Results show that at a range of low FNA concentrations (0.24-2.25 mg HNO_2-N L^{-1}) TAG recovery was strongly enhanced with increasing FNA concentration. An FNA concentration of around 2 mg HNO_2-N L^{-1} resulted in a 3.3-fold increase in fatty acid recovery over untreated algae, but higher FNA concentrations (13.49 and 26.98 mg HNO_2-N L^{-1}) were detrimental to TAG recovery. Analysis of the fatty acid profile revealed that the higher FNA concentrations caused a reduction in polyunsaturated fatty acids. Also, the ratio of extracted fatty acids to total lipids was significantly reduced when high FNA concentration were applied, and only non-fatty acid lipids potentially benefited from more intense FNA pre-treatments.

Abstract

Fatty acid (FA) recovery from algal biomass is primarily limited by the rigid algal cell envelope (cell wall and cell membrane). In this work, the effect of FNA pre-treatment on triacylglyceride (TAG) fatty acids recovery from algal biomass with six different FNA concentrations is reported. Results show that at a range of low FNA concentrations (0.24-2.25 mg HNO_2-N L^{-1}) fatty acid recovery was enhanced dramatically with increasing FNA concentration but then higher FNA concentrations (13.49 and 26.98 mg HNO_2-N L^{-1}) were detrimental to TAG FA recovery. Analysis of the fatty acid profile revealed that the higher FNA concentrations caused reduction in polyunsaturated fatty acids (PUFA). Also, the ratio of extracted fatty acids to total lipids is significantly reduced when high FNA concentration is applied, as only non-fatty acid lipids potentially remain to benefit from more intense FNA pre-treatment. Optimized FNA concentration was determined as around 2 mg HNO_2-N L^{-1} which resulted in a 3.3-fold increase in fatty acid recovery over untreated algae.

Keywords: Free nitrous acid; Algae; Fatty acid; Triacylglyceride

1. Introduction

It was recently shown that total lipid (TL) extraction can be enhanced dramatically by pre-treatment with FNA (Bai et al., 2014). However, TL is a lumped measure of triacylglycerides (TAGs), fatty acids, glycerolipids, as well as other "solvent-philic" compounds such as photosynthesis pigments. Total lipid is an indicator of the content of valuable inclusions, but it is not a direct measure of them. In making algal biodiesel, it is the fatty acid content associated with TAGs that matters since these fatty acids are the feedstock which react with methanol in a reaction known as transesterification to produce fatty acid methyl esters (FAME) (Hu et al., 2008).

Moreover, the properties of TAGs, and consequently of the biodiesel fuel, are determined by the fatty acid profile of the feedstock (Bajpai & Tyagi, 2006; Francisco et al., 2010; Knothe, 2005). For example, Ramos *et al.* (2009) investigated the biodiesel properties derived from ten refined vegetable oils and found some critical properties like cetane number (CN), heat of combustion (HG), and oxidative stability were correlated with the fatty acid composition of each oil, with the most important parameters being: degree of unsaturation (DU) and long chain saturated factor (LCSF). Therefore, quantifying fatty acids amongst total lipids is essential to determine the TAG properties.

In addition to being useful for generating biodiesel, algal fatty acids are of value as a food supplement. Of particular interest are long-chain PUFA. Among PUFA produced by algae, some have been proven to be especially beneficial to our diet, including eicosapentaenoic acid (EPA, C20:5ω3), docosahexaenoic acid (DHA, C22:6 ω3), and arachidonic acid (ARA, C20:4 ω6) (Arts et al., 2001). These fatty acids are termed "essential fatty acids (EFAs)".

Since TAG fatty acid recovery is often limited by the low extraction yield from the cells, effective pre-treatment techniques are called for (Jin et al., 2012; Lee et al., 2010; Sheng et al., 2011). However, although most of the reported pre-treatment techniques can enhance total lipid recovery, the amount of TAG fatty acids among the lipids recovered and the characteristics of the recovered fatty acids vary due to the different pre-treatment mechanisms. Some of the techniques, such as pulsed-electric-field pre-treatment (Sheng et al., 2011), do not have any obvious effect on the fatty acid profile. However some other pre-treatment techniques can greatly affect the fatty acid profile. Sharma *et al.* (2014) reported that UV-C could increase the proportion of unsaturated fatty acids (USFAs) in the recovered lipids, and other researchers found microwave-assisted lipid extraction could produce higher antioxidant-containing lipids, i.e. USFAs (Balasubramanian et al., 2011; El-Abassy et al., 2010). Lipids are inherently susceptible to oxidation through multiple pathways, with their stability being dependent on the specific fatty acids present, environmental conditions, and the sample matrix. Shahidi and Zhong (2010) revealed that elevated temperatures can accelerate the auto oxidation process and some enzymes can also specifically catalyse the oxidation of PUFA. It is therefore essential to examine effect of algae pre-treatment on TAG fatty acid recovery.

Exposure to free nitrous acid (FNA) is a potential pre-treatment technology that has been trialled for improving total lipid extraction from algae (Bai et al., 2014). As discussed above, data on the amount TAG fatty acid and the characteristics of the fatty acid profile are vital for the production of biodiesel and EFAs. Therefore, in this work, the effect of FNA pre-treatment on TAG fatty acid recovery from algal biomass is reported. Six different FNA concentrations are considered. The relationship between TAG fatty acid recovery and FNA concentration was assessed. Additionally, the effect of FNA pre-treatment on different types of fatty acids was evaluated, with the view to identifying the optimum FNA concentration for lipid and fatty acid recovery.

2. Materials and Methods

2.1. Algae collection and FNA pre-treatment operation

Tetraselmis striata M8, which is a marine algae with high lipid accumulation ability, was cultured in the University of Queensland. Lipids accumulation in algal cells was stimulated by nitrogen

starvation. After algae cultivation and lipids accumulation, algal biomass was harvested by centrifuged at 3270 g (Beckman Coulter, Allegra™ X-12) for 3 min (Beckman Coulter, Allegra™ X-12).

Table 1 summarize the FNA pre-treatment conditions and Figure 1 represents the experimental operation diagram. The harvested algal paste was re-suspended with de-ionized water and then the mixed liquor was evenly distributed between four beakers (reactor volume 500 ml) for FNA pre-treatment. FNA concentration in each test was varied by controlling the initial dosage of sodium nitrite and monitoring pH, which was kept constant during the whole pre-treatment at 5.0±0.2 or 6.0±0.2 (according to Table 1) through manually adding 0.5 M HCl. The FNA concentration can be calculated using the following formula. Detailed experimental set-ups and FNA pre-treatment procedure can be found in Bai *et al.* (2014).

$$FNA(mg\ HNO_2\text{-}N/L) = S_{NO2^- - N}/(K_a \times 10^{pH}) \qquad \text{Eq. 1}$$

where $S_{NO2^- - N}$ as dissolved NO_2^- concentration (mg NO_2-N/L) and $K_a = e^{-2{,}300/(273.15+T)}$ at an operation temperature T (º C) (25ºC in this study) (Anthonisen et al., 1976).

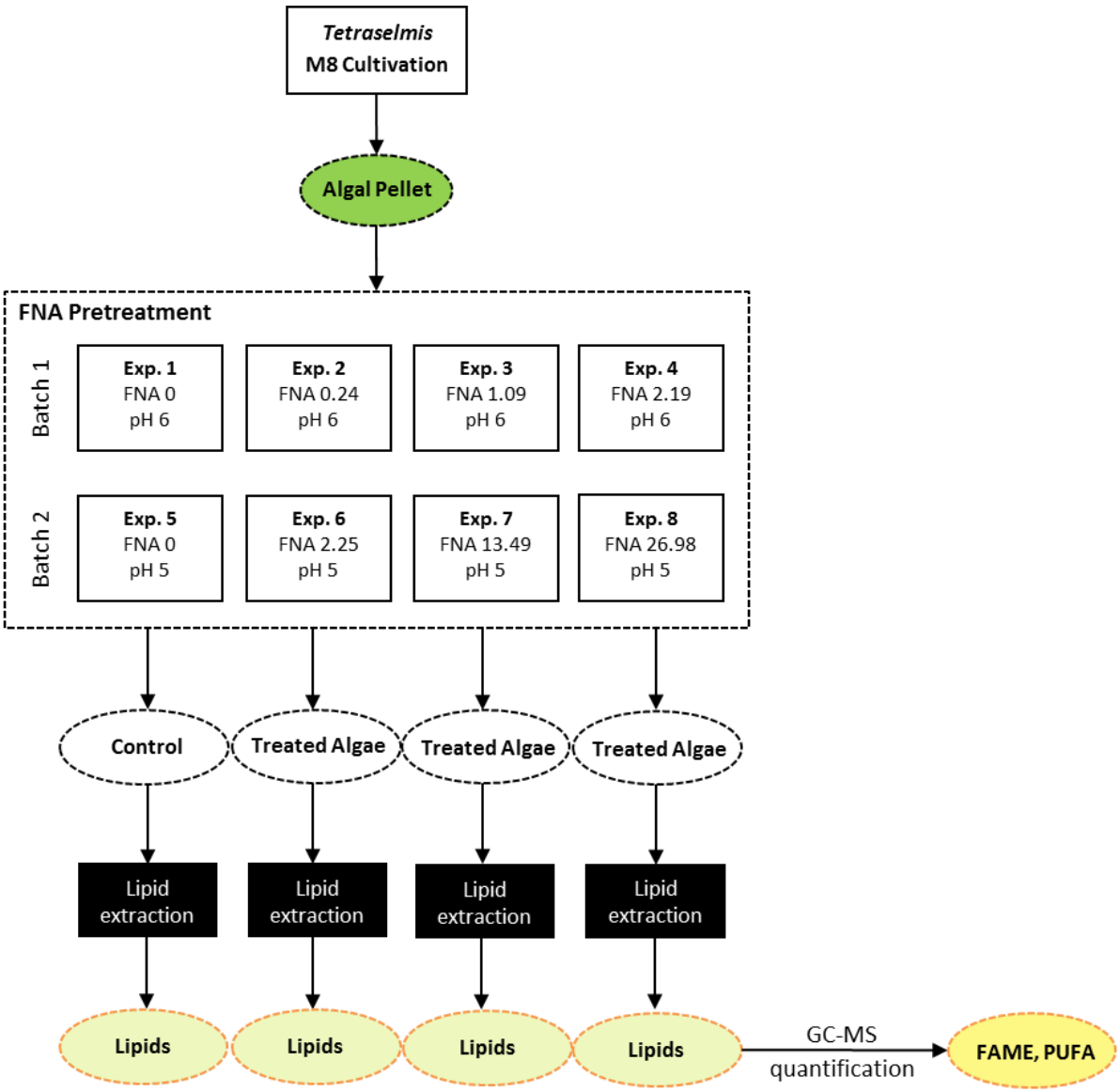


Figure 1: A flow diagram of experimental design and operation.

Table 1: Experimental conditions applied in batch tests.

	Batch1				**Batch 2**			
	Exp. 1	**Exp. 2**	**Exp. 3**	**Exp. 4**	**Exp. 5**	**Exp. 6**	**Exp. 7**	**Exp. 8**
pH	6	6	6	6	5	5	5	5
NO_2^- (mg N/L)	0	100	450	900	0	100	600	1200
FNA (mg HNO_2-N/L)	0	0.24	1.09	2.19	0	2.25	13.49	26.98

2.2. Total lipid extraction, quantification and fatty acid characterisation

Total lipid extraction, quantification

Lipids were extracted by chloroform : methanol (1:1, v/v) with the collected wet algal biomass using Bligh and Dyer method (1959). 3 g of wet algal paste was treated with 20 ml of solvent, followed by adding 10 ml de-ionised water. The bottom chloroform layer of the extracts was dried in vacuum until constant weight. Moisture of algal pastes was determined by weighing certain amount of wet paste on filter paper (GF/C, Whatman) and dried in 105 °C oven overnight, all in triplicates.

Fatty acid characterisation

2 mg of the extracted lipids were re-dissolved in 2 mL chloroform and a 100 µL aliquot was taken and dried down. Then the lipids were hydrolyzed and methyl-esterified with 300 µL of 2% H_2SO_4 in methanol solution at 80 °C by shaking (480 rpm) for 2 h on a thermal-mixture. Prior to the esterification, 50 µg of heneicosanoic acid (C21) was added to the pellet in each sample as an internal standard. After esterification, 300 µL of 0.9% (w/v) NaCl and 300 µL of HPLC grade hexane were added and vortexed for 20 s. Phase separation was performed by centrifugation at 16,000 x g for 3 min and the hexane layer was used for fatty acid methyl ester profile analysis by GC-MS. Using 1 µL on a splitless injection system, GC-MS analyses were carried out on an Agilent 6890 GC coupled to a 5975 MSD. A DB-Wax column (Agilent, 122-7032) was used with running conditions as described in Agilent's RTL DBWax method (Application note: 5988-5871EN). Identification of fatty acid methyl esters was based on mass spectral profiles and retention times in the Agilent's RTL DBWax method.

The cetane number (CN) and the degree of unsaturation (DU) were determined by empirical equations (Eq 2 and Eq 3). Data from the literature were also used (Munack, 2006).

$$CN = 46.3 + \frac{5458}{SV} - 0.125 * IV \qquad \text{Eq. 2}$$

$$DU = MUFA + (2 \times PUFA) \qquad \text{Eq. 3}$$

where saponification value $(SV) = \sum \frac{(560 \times N)}{M}$, iodine value $(IV) = \sum \frac{(254 \times D \times N)}{M}$, D is the number of double bonds, M is the molecular mass and N is the percentage of each fatty acid component. MUFA and PUFA are their percentage of total fatty acids (%wt of total fatty acids).

3. Results

Already published data shows that after FNA pre-treatment, total lipids, as quantified by the Bligh and Dyer method, increases with pre-treatment time (up to 48 h) and FNA concentration (up to 2.19 mg HNO_2-N L^{-1}) (Bai et al., 2014). In this work, GC-MS analysis was performed to quantify the TAG fatty acid contribution to those lipids and characterise the fatty acid profile. Table 2 presents the characteristics of fatty acids in the recovered lipids, including the summary of different groups of fatty acids such as saturated fatty acids (SFA), USFA, and MUFA and PUFA.

As shown in Table 2, for untreated algae, 52 % of the total recovered lipids were TAG fatty acids, including C16 (palmitic, 20.2 %), C16:4 (hexadecatetraenoic, 16.8 %), C18:2 (linoleiaidic, 14.9 %), C18:3 (linolenic, 26.9 %) and C20:4 (eicosatetraenoic, 10.6 %). The portions of SFA and USFA were 14.6 % and 79.7 % among total FA respectively.

As shown in Figure 2, for a range of low FNA concentration pre-treatments (0.24-2.25 mg HNO_2-N L^{-1}), an increase in FNA concentration boosted TAG fatty acid recovery along with total lipid recovery. A maximum 3.3-fold enhancement in TAG fatty acid recovery from FNA-2.19 over the untreated algae was observed. However, although higher FNA concentration (13.49 and 26.98 mg HNO_2-N L^{-1}) remained effective for recovery of total lipids, it was detrimental for TAG fatty acid recovery. With 13.49 and 26.98 mg HNO_2-N L^{-1} FNA pre-treatment, TAG fatty acid recovery was significantly lower than for low range FNA pre-treatment and only slightly higher than it was for untreated algae. Optimum FNA concentration was around 2 mg HNO_2-N L^{-1} for the TAG fatty acid recovery.

The properties of biodiesel that are related to the FA profile include CN and oxidative stability (measured as the degree of unsaturation (DU)). CN is a dimensionless descriptor of the ignition quality of diesel. The CN value of the reference fuel cetane is 100. Standard ASTM D6751 (Testing & Materials, 2009) for biodiesel fuel requires a minimum CN of 47. As shown in Table 2, the CN value of the TAG fatty acids recovered improved to >47 after FNA pre-treatment, comparing to 44 in untreated algae. The degree of unsaturation (DU) is another measure of the physical and fuel properties of the biodiesel. The values obtained varied from 112.5 to 147.0 after different levels of FNA pre-treatment, which were lower than, and therefore an improvement over, the control (DU=153.9).

Table 2: Total lipids (TL), total fatty acid (FA), total saturated fatty acid (SFA), total unsaturated fatty acid (USFA), total mono unsaturated fatty acid (MUFA), and total poly unsaturated fatty acid (PUFA), and biodiesel quality parameters. All the fatty acid data is shown as wt % of dry algal biomass, and crude lipids data are shown as mean (± error), n=2.

	Untreated algae	**pH6-FNA0**	**pH6-FNA0.24**	**pH6-FNA1.09**	**pH6-FNA2.19**	**pH5-FNA0**	**pH5-FNA2.25**	**pH5-FNA13.49**	**pH5-FNA26.98**
FA	4.73	7.22	9.24	12.60	15.62	7.62	13.89	9.63	9.73
SFA	0.96	1.67	2.19	2.47	3.26	1.71	3.14	3.12	3.42
USFA	3.77	5.55	7.05	10.13	12.36	5.91	10.75	6.51	6.31
MUFA	0.25	0.96	0.96	1.31	1.81	0.91	1.64	1.53	1.73
PUFA	3.52	4.59	6.09	8.82	10.55	5.00	9.11	4.98	4.58
TL*	9.01 (±0.6)	13.23(±0.2)	15.58 (±0.4)	18.96 (±0.1)	21.75 (±0.3)	18.77 (±0.5)	23.54 (±0.2)	23.84 (±0.3)	24.35 (±0.3)
CN	44.4	49.9	47.2	47.1	47.0	48.6	48.8	54.3	56.1
DU	153.9	131.1	142.1	145.5	147.0	144.4	143.8	119.4	112.5
C16	0.96	1.67	2.19	2.47	3.26	1.71	3.14	3.12	3.42
C16:1	0.04	0.28	0.23	0.31	0.42	0.15	0.25	0.22	0.31
C16:2	0.00	0.01	0.00	0.00	0.04	0.06	0.08	0.00	0.04
C16:3	0.25	0.21	0.36	0.49	0.58	0.65	1.10	0.75	0.41
C16:4	0.79	0.73	1.26	1.69	1.94	0.92	1.70	0.53	0.51
C18:1	0.15	0.64	0.69	0.94	1.29	0.64	1.05	1.07	1.20
C18:2	0.71	0.91	1.33	1.94	2.35	1.73	3.25	2.29	2.35
C18:3	1.27	1.16	2.04	3.33	3.99	1.17	2.20	1.13	1.15
C20:1	0.00	0.00	0.00	0.00	0.00	0.07	0.17	0.10	0.12
C20:4	0.50	1.57	1.10	1.37	1.65	0.47	0.78	0.28	0.12
C22:1	0.06	0.04	0.04	0.06	0.10	0.05	0.17	0.14	0.10

* Data from Bai *et al.* (2014).

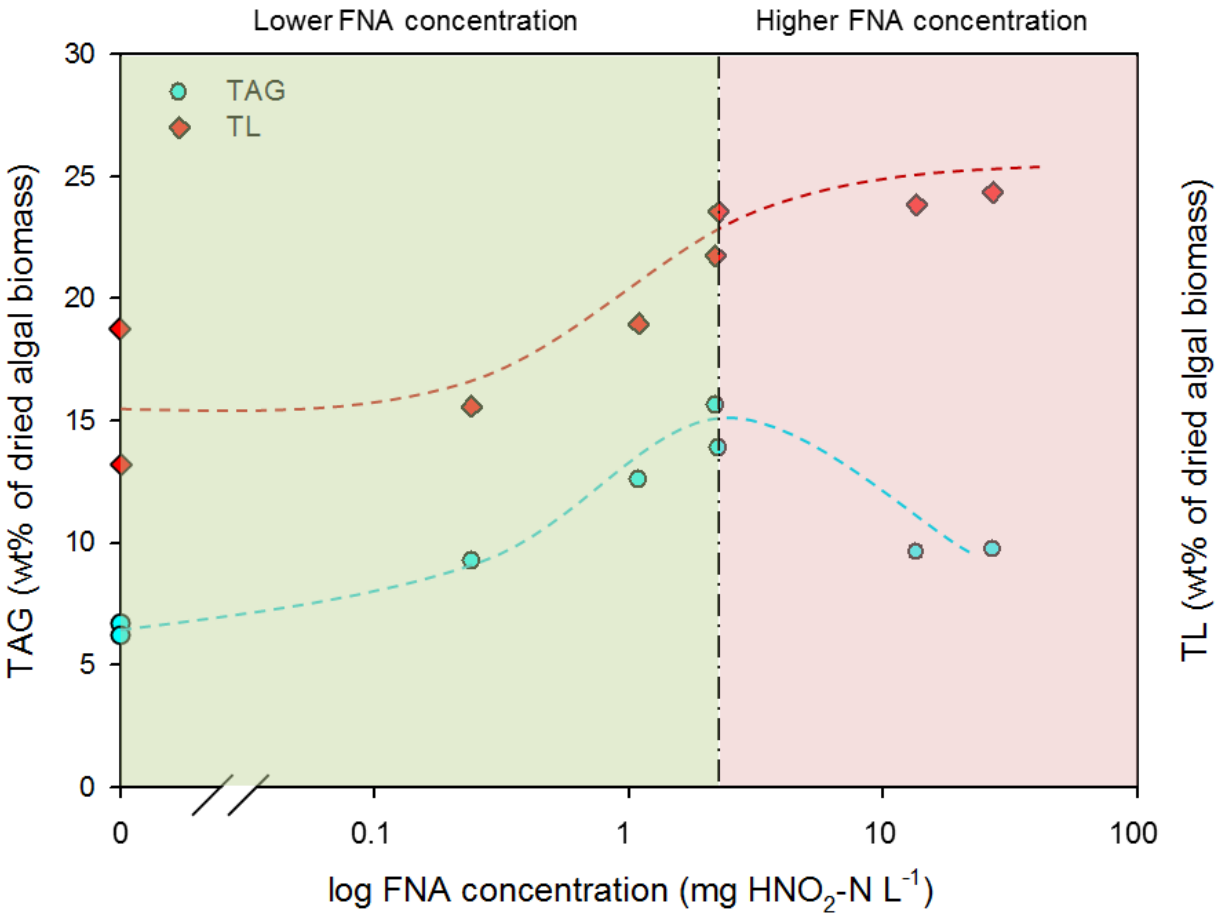


Figure 2: Total lipid (TL) and TAG fatty acid recovery after different levels of FNA pre-treatment.

After FNA pre-treatment, the major detected fatty acids did not change, but the relative amounts of each fatty acid varied. As shown in Figure 3 (A-B), the amount of SFA and MUFA was enhanced with increasing FNA concentration (from 0 to 2.25 mg HNO_2-N L^{-1}) until a plateau was reached (at > 2.25 mg HNO_2-N L^{-1}). This trend is consistent with the data on total lipid recovery. However, the absolute amount of PUFA in the recovered lipids showed a peak at a FNA concentration around 2 mg HNO_2-N/L (Figure 3C); higher FNA pre-treatments (13.49 and 26.98 mg HNO_2-N L^{-1}) dramatically reduced the PUFA recovery.

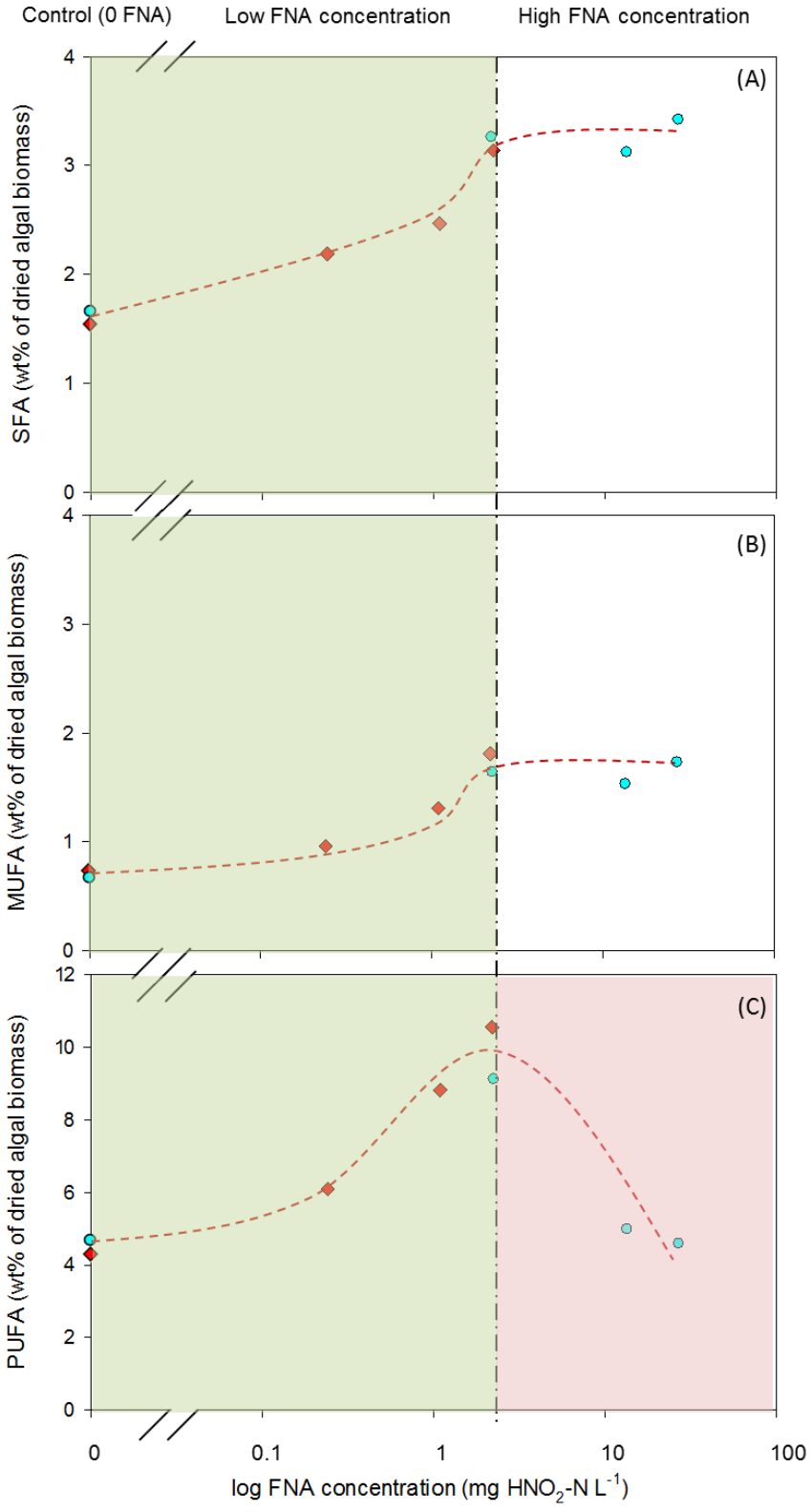


Figure 3: Different types of fatty acids recovery after FNA pre-treatment. Red diamonds show results of Batch 1, cyan circles show results of Batch 2.

4. Discussion

TAG fatty acid recovery

For a range of low FNA concentrations, FNA pre-treatment boosts TAG fatty acid recovery even beyond the general boost to total lipid recovery. However, although exposure to higher FNA concentration at least maintained recovery of total lipids, it was detrimental for the TAG fatty acid recovery. As discussed by Bai *et al.* (2014), FNA pre-treatment leads to cell disruption. In this study, cell disruption was characterised by protein and polysaccharides release (Figure 4), and improved access to TAG fatty acids. But significantly, Figure 4 shows the release of protein and polysaccharides as a function of FNA concentration was markedly different, with protein being released after exposure to low FNA concentration but polysaccharides mainly being released after exposure to high FNA concentration.

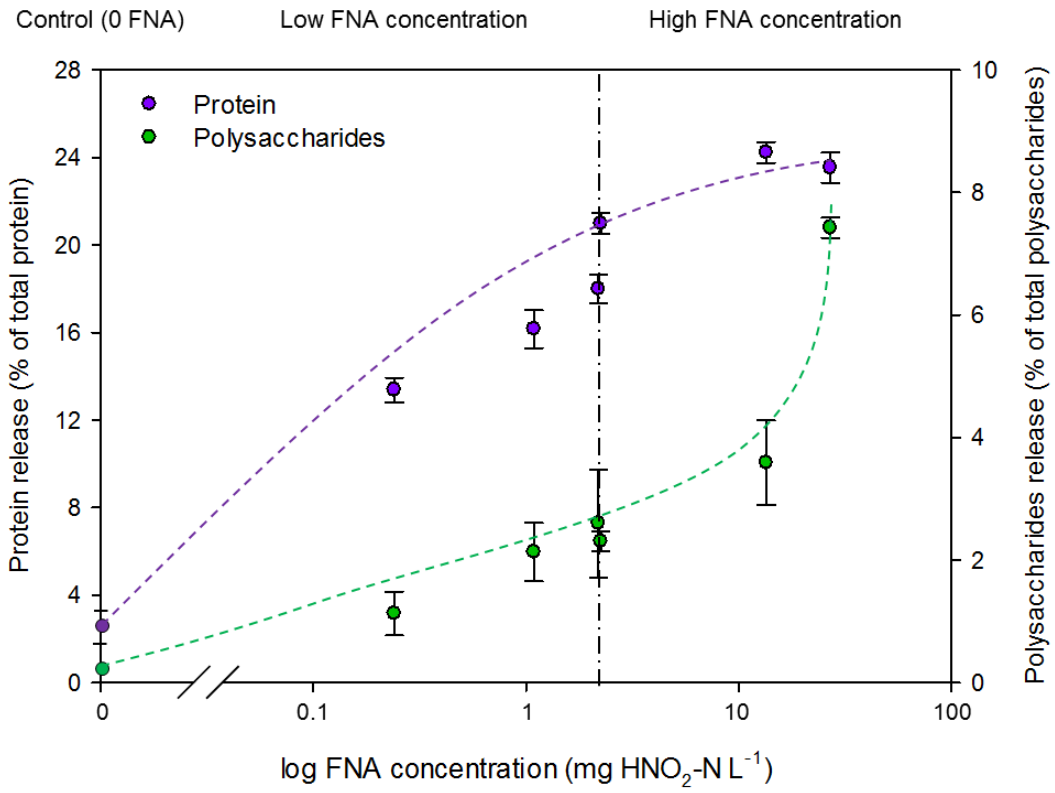


Figure 4: Soluble protein and polysaccharides released and dissolved into the pre-treatment liquor after FNA pre-treatment. All the data has been normalized to the proportion of released component to total component content in the culture.

For single-cell green algae, such as *Tetraselmis* sp. applied in this study, most of the protein content

of the biomass belongs to the intracellular components, whereas polysaccharides are the main constituent of the cell wall and intracellular starch components (such as storage starch granules and shell starch grains surrounding pyrenoids) (Gibbs, 1962). Exposure to low FNA concentration apparently led to minor cell disruption, sufficient to release protein and improve access to TAG fatty acids. Exposure to high FNA concentration apparently led to deep penetration into chloroplasts and membrane lipids (i.e. phospholipids), characterised by relatively high polysaccharide mobilisation and potentially elevated recovery of non-TAG fatty acids, which was countered by oxidation of some TAG fatty acids (discussed further in the following section) Exposure to very high FNA concentration (> 20 mg HNO_2-N L^{-1}) resulted only in solubilisation of intracellular polysaccharides. In this work, the optimum FNA concentration was around 2 mg HNO_2-N L^{-1}, but one should note that the optimum could be species dependent as different algal species may have varying cell ultrastructures (Pragya et al., 2013). For example, in the study operated by Prabakaran and Ravindran (2011), osmotic shock pre-treatment method was more effective on *Tolypothrix* sp. than on *Nostoc* sp, with an increase on lipid extraction yield of 3 times and 1.5 time, respectively.

TAG fatty acid characteristics

The fatty acid profile post 2 mg HNO_2-N L^{-1} was typical of this algae (Sharma et al., 2014). The four main fatty acids were C16, C16:4, C18:2 and C18:3. The portions of USFA to SFA were 20.3 % and 79.7 % among total TAG fatty acids respectively. The main difference compared to previous studies was the abundance of C20:4 and Essential Fatty Acid (EEA), which was quantified as one of the major fatty acids in this study whereas it was only a minor contributor to fatty acid composition in the previous published work (Sharma et al., 2014). This could be due to different culture conditions such as light, nutrient condition or different harvest stage (Brown et al., 1996).

The FNA concentration affected the fatty acid profile. As seen from Figure 3 (A-C), for a range of low FNA pre-treatments (0.24-2.25 mg HNO_2-N L^{-1}), the amount of SFA, MUFA and PUFA being recovered was boosted with increasing FNA concentration. However, higher range FNA pre-treatments (13.49 and 26.98 mg HNO_2-N L^{-1}) showed negative effect on fatty acids recovery (Figure 2). As shown in Table 2, with higher range FNA pre-treatments (13.49 and 26.98 mg HNO_2-N L^{-1}), although there was an enhancement in C16, C18:1, C18:2 and C22:1 recovery over untreated algae, the recovery of the other fatty acids was largely reduced, especially the recovery of polyunsaturated fatty acids (eg, C16:3, C16:4 and C20:4). It has been reported that active groups of FNA ionized system such as NO• (nitric oxide) and NO_2 can induce lipid peroxidation (Hogg &

Kalyanaraman, 1999). Therefore, it is very likely that the reason for the negative effect of high FNA concentrations (13.49 up to 26.98 mg HNO_2-N L^{-1}) on PUFA recovery is because that unsaturated fatty acids were damaged or oxidised by NO• and NO_2.

The biodiesel that could be obtained from the TAG fatty acid recovered was improved by FNA pre-treatment in several aspects. As shown in Table 2, the CN values of TAG fatty acid recovered from FNA pre-treated algae are increased in accordance with the standard ASTM D6751. Besides, lower DU value after FNA pre-treatments can result in an increased oxidation stability of biodiesel (Knothe et al.) and a decrease the NO_X exhaust emissions (McCormick et al., 2001).

In addition to generating biodiesel, the essential fatty acids (EFAs) as valuable food supplement are of particular interest (Arts et al., 2001). Comparing to untreated algae, C20:4 (arachidonic, ω6) recovery kept increasing as FNA concentration increased up to 2.25 mg HNO_2-N L^{-1}. The highest C20:4 was detected as 1.65 (wt% of dry algal biomass), which was a 3.3 times increase than the untreated algae.

5. Conclusions

In general, the quantity of TAG fatty acid recovered and the quality of final product that could be obtained (biodiesel or food supplement) were both enhanced by FNA pre-treatment. Detailed fatty acid profile reveals that at a range of low FNA concentrations (0.24-2.25 mg HNO_2-N L^{-1}), TAG fatty acid recovery was enhanced dramatically with increasing FNA concentration but then higher FNA concentrations (13.49 and 26.98 mg HNO_2-N L^{-1}) were detrimental to FA recovery. Also, the ratio of extracted fatty acids to total lipids is significantly reduced when high FNA concentration is applied. Optimized FNA concentration was determined as around 2 mg HNO_2-N L^{-1} for TAG fatty acid recovery.

Acknowledgements

The authors wish to thank Mr. Rakesh Narala of the Algae Biotechnology Group (The University of Queensland, Australia), for providing the algal inoculum and culturing protocols.

Reference

Anthonisen, A.C., Loehr, R.C., Prakasam, T.B.S., Srinath, E.G. 1976. Inhibition of Nitrification by Ammonia and Nitrous Acid. Journal (Water Pollution Control Federation), 48(5), 835-852.

Arts, M.T., Ackman, R.G., Holub, B.J. 2001. " Essential fatty acids" in aquatic ecosystems: a crucial link between diet and human health and evolution. Canadian Journal of Fisheries and Aquatic Sciences, 58(1), 122-137.

Bai, X., Ghasemi Naghdi, F., Ye, L., Lant, P., Pratt, S. 2014. Enhanced lipid extraction from algae using free nitrous acid pretreatment. Bioresource Technology(0).

Bajpai, D., Tyagi, V.K. 2006. Biodiesel: Source, Production, Composition, Properties and Its Benefits. Journal of Oleo Science, 55(10), 487-502.

Balasubramanian, S., Allen, J.D., Kanitkar, A., Boldor, D. 2011. Oil extraction from Scenedesmus obliquus using a continuous microwave system – design, optimization, and quality characterization. Bioresource Technology, 102(3), 3396-3403.

Bligh, E., Dyer, W. 1959. A rapid method of total lipid extraction and purification. Can. J. Biochem. Physiol, 37(8), 911-917.

Brown, M.R., Dunstan, G.A., Norwood, S.J., Miller, K.A. 1996. Effects of harvest stage and light on the biochemical composition of the diatom Thalassiosira Pseduonana. Journal of Phycology, 32(1), 64-73.

El-Abassy, R.M., Donfack, P., Materny, A. 2010. Assessment of conventional and microwave heating induced degradation of carotenoids in olive oil by VIS Raman spectroscopy and classical methods. Food Research International, 43(3), 694-700.

Francisco, É.C., Neves, D.B., Jacob-Lopes, E., Franco, T.T. 2010. Microalgae as feedstock for biodiesel production: Carbon dioxide sequestration, lipid production and biofuel quality. Journal of Chemical Technology & Biotechnology, 85(3), 395-403.

Gibbs, S.P. 1962. The ultrastructure of the pyrenoids of green algae. Journal of Ultrastructure Research, 7(3–4), 262-272.

Hogg, N., Kalyanaraman, B. 1999. Nitric oxide and lipid peroxidation. Biochimica et Biophysica Acta (BBA) - Bioenergetics, 1411(2–3), 378-384.

Hu, Q., Sommerfeld, M., Jarvis, E., Ghirardi, M., Posewitz, M., Seibert, M., Darzins, A. 2008. Microalgal triacylglycerols as feedstocks for biofuel production: perspectives and advances. The Plant Journal, 54(4), 621-639.

Jin, G., Yang, F., Hu, C., Shen, H., Zhao, Z.K. 2012. Enzyme-assisted extraction of lipids directly from the culture of the oleaginous yeast Rhodosporidium toruloides. Bioresource Technology, 111(0), 378-382.

Knothe, G. 2005. Dependence of biodiesel fuel properties on the structure of fatty acid alkyl esters. Fuel Processing Technology, 86(10), 1059-1070.

Knothe, G., Krahl, J., Gerpen, J.V. Biodiesel Handbook (2nd Edition), AOCS Press.

Lee, J.-Y., Yoo, C., Jun, S.-Y., Ahn, C.-Y., Oh, H.-M. 2010. Comparison of several methods for effective lipid extraction from microalgae. Bioresource Technology, 101(1, Supplement), S75-S77.

McCormick, R., Graboski, M., Alleman, T., Herring, A. 2001. Impact of biodiesel source material and chemical structure on emissions of criteria pollutants from a heavy-duty engine. . Environmental Science and Technology, 35, 1742-1747.

Munack, A. 2006. Biodiesel – A comprehensive handbook. Martin Mittelbach, Claudia Remschmidt (Ed.). WILEY-VCH Verlag.

Prabakaran, P., Ravindran, A.D. 2011. A comparative study on effective cell disruption methods for lipid extraction from microalgae. Letters in Applied Microbiology, 53(2), 150-154.

Pragya, N., Pandey, K.K., Sahoo, P.K. 2013. A review on harvesting, oil extraction and biofuels production technologies from microalgae. Renewable and Sustainable Energy Reviews, 24(0), 159-171.

Ramos, M.J., Fernández, C.M., Casas, A., Rodríguez, L., Pérez, Á. 2009. Influence of fatty acid composition of raw materials on biodiesel properties. Bioresource Technology, 100(1), 261-268.

Shahidi, F., Zhong, Y. 2010. Lipid oxidation and improving the oxidative stability. Chemical Society Reviews, 39(11), 4067-4079.

Sharma, K., Li, Y., Schenk, P. 2014. UV-C-mediated lipid induction and settling, a step change towards economical microalgal biodiesel production. Green Chemistry.

Sheng, J., Vannela, R., Rittmann, B.E. 2011. Evaluation of Cell-Disruption Effects of Pulsed-Electric-Field Treatment of Synechocystis PCC 6803. Environmental Science & Technology, 45(8), 3795-3802.

Testing, A.S.f., Materials. 2009. ASTM D6751-09, Standard Specification for Biodiesel Fuel Blend Stock (B100) for Middle Distillate Fuels. ASTM West Conshohocken, PA.

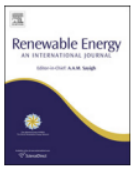
Appendix E

Enhanced methane production from algae using free nitrous acid pre-treatment

Contents lists available at ScienceDirect

Renewable Energy

journal homepage: www.elsevier.com/locate/renene



Elsevier Editorial System(tm) for Renewable Energy
Manuscript Draft

Manuscript Number: RENE-D-14-02112

Title: Enhanced methane production from algal digestion using free nitrous acid pre-treatment

Article Type: Original Research Paper

Keywords: Free nitrous acid; Algae; Anaerobic digestion; Biogas; Inhibition

Corresponding Author: Dr. Steven Pratt, Ph.D.

Corresponding Author's Institution: University of Queensland

First Author: Xue Bai

Order of Authors: Xue Bai; Paul A Lant; Paul D Jensen; Sergi Astals; Steven Pratt, Pd.D

Abstract: The methane yield from the digestion of algae is typically much lower than the theoretical methane yield, and lower than yields reported for other organic substrates. This study presents a novel free nitrous acid (FNA) pre-treatment technique to improve methane production from algal biomass. The methane production yield through anaerobic digestion was found to be dramatically enhanced by FNA pre-treatment (2.31 mg HNO2-N L-1), with a 53 % increase in the methane yield (from 161 to 250 L CH4 per kg VS added). A two substrate model was used to describe the apparent presence of rapid and slowly degradable material. Model-based analysis revealed that with FNA pre-treatment (2.31 mg HNO2-N L-1), the availability of both rapid and slowly biodegradable substrates were increased. Higher levels of nitrite (159 and 1006 mg N L-1) had an inhibitory/toxic effect. For this reason, coupled with the fact that denitrification of nitrite consumes organic substrate, it is concluded that pre-treatment liquor should be removed before digestion.

Abstract

Anaerobic digestion (AD) of algal biomass is an essential component of algal biofuel production systems. However, the methane yield from digestion of algae is typically much lower than the theoretical methane yield, and lower than yields reported for other organic substrates. This study presents a novel free nitrous acid (FNA) pre-treatment technique to improve methane production from algal biomass. The methane production yield through anaerobic digestion was found to be dramatically enhanced by FNA pre-treatment (2.31 mg HNO_2-N L^{-1}), with a 53 % increase in the methane yield (from 161 to 250 L CH_4 per kg VS). A two substrate model was used to describe digestion to account for the apparent presence of rapid and slowly degradable material. Model-based analysis revealed that with FNA pre-treatment (2.31 mg HNO_2-N L^{-1}), the availability of both rapid and slowly biodegradable substrates were increased. Higher levels of FNA (159 and 1006 mg N L^{-1}) had an inhibitory/toxic effect. For this reason, coupled with the fact that denitrification of nitrate consumes substrate, it is concluded that pre-treatment liquor should be removed before digestion.

Keywords: Free nitrous acid; Algae; Anaerobic digestion; Biogas; Inhibition

1. Introduction

Anaerobic digestion (AD) of algal biomass will be a necessary step to make the algal biofuel production feasible (Sialve et al., 2009). However, methane yields for algae digestion are often relatively low (Richmond, 2004). A couple of factors may contribute to reduced yields, including the physical characteristics of algae as well as the inhibitory effects of digestion by-products such as ammonia (Chen et al., 2008; Ras et al., 2011). In particular, the cell envelope (cell wall and membrane) of algae is described as a rigid barrier and therefore limits access for biological activity.

A number of algae pre-treatment techniques to potentially increase methane yields have been investigated, including thermal hydrolysis, ultrasound, microwave, chemical and enzyme hydrolysis methods, etc. (Cho et al., 2013; Keymer et al., 2013; Mahdy et al.; Passos et al., 2014). These technologies destroy cell structures and release intracellular and/or extracellular constituents to the liquid phase. The disrupted cells and released constitutes are more readily biodegraded during digestion, therefore enhancing methane production yield. However, all of the above pre-treatment techniques have high energy and/or chemical requirements (Singh & Olsen, 2011).

A potential alternate pre-treatment technique that has already been demonstrated to be effective for improving digestibility of waste activated sludge (WAS) from wastewater treatment plants (WWTP) is free nitrous acid (FNA) pre-treatment (Wang et al., 2013). When digesting WAS from wastewater treatment plants, FNA pre-treatment (at 1.78-2.13 mg HNO_2-N L^{-1}) leads to an improved methane potential, with the highest improvement being approximately 27 % (from 201 to 255 L CH_4 per kg VS added) compared to untreated WAS (Wang et al., 2013). FNA pre-treatment could be applicable for algae, especially considering that our recent study on FNA pre-treatment to disrupt algae to boost lipid extraction showed that FNA, at parts per million (ppm) levels, can severely disrupt algal cells (Bai et al., 2014).

In this paper we aim to quantify for the first time the gain in methane yield and production rate that can be achieved by applying FNA pre-treatment to algae, and to understand the pre-treatment mechanism. Both untreated algae and FNA treated algae are tested. It is worth noting that nitrite present in the FNA pre-treatment liquor is a concern due to its potential toxicity to methanogenesis (Banihani et al., 2009; Chen et al., 2008). Thus the effect of different levels of nitrite present in anaerobic digestion of algae (from FNA pre-treatment liquor) was also evaluated.

2. Materials and Methods

2.1. Microalgae harvest and sludge source

Tetraselmis striata M8 was cultured in an open algal culture pond at The University of Queensland. F/2 medium was used as the growth media. Culture pH was kept constant at 8.5±0.2 by CO_2 injection with an electronic controller, and the depletion of nutrients ($NO_3^-$and PO_4^{3-}) was tested using seawater aquaria nutrient kits (DAPI Aquarium Pharmaceuticals for NO_3^- and Nutrafin for PO_4^{3-}). The algae was grown to a dry weight of around 1 g L^{-1} and then 300 L algal culture was concentrated to a paste by centrifugation at 3270 g (Beckman Coulter, Allegra™ X-12) for 3 min in 6 L batches. The algae paste was collected and re-dissolved into de-ionized water (DI water) prior to FNA pre-treatment (described in Section 2.2.).

The inoculum for the biochemical methane potential (BMP) tests (described in Section 2.3.) was harvested from a mesophilic anaerobic digester treating mixed primary sludge in the wastewater treatment plant (WWTP) (Queensland, Australia).

Total chemical oxygen demand (TCOD), soluble chemical oxygen demand (SCOD), total solids (TS), volatile solids (VS), of the algae and inoculum are shown in Table 1 (standard errors obtained through triplicate measurements). The composition of the algae was also measured.

Table 1: Characteristics of algae and inoculum used in BMP tests, mean±SE (n=3).

Characteristics	**Algae**	**Inoculum**
pH	-	7.5±0.1
TCOD (g L^{-1})	11.5±0.05	24.8±0.2
SCOD (g L^{-1})	2.39±0.1	0.54±0.05
TS (g L^{-1})	9.80±0.3	24.5±0.1
VS (g L^{-1})	7.40±0.6	14.4±0.1
Protein (% of dry weight)	49.4±0.2	-
Carbohydrates (% of dry weight)	16.0±0.3	-
Lipids (% of dry weight)	6.40±0.2	-
Ash (% of dry weight)	24.5±0.5	-

-: not available.

2.2. FNA pre-treatment

FNA pre-treatment comprised three experiments and a control. The experimental conditions are summarised in Table 2. The algae paste was re-dissolved into DI water and then the mixed liquor was evenly distributed between four beakers (reactor volume 1 L).

Pre-determined amounts of sodium nitrite stock solution (30 g N L^{-1}) were added to the batch reactors in different volumes at the beginning of each experiment, which resulted in initial concentrations of nitrite varying between 0 to 1900 mg HNO_2-N L^{-1}. pH was kept approximately constant through pre-treatment at 5.5±0.2 by manually adding 0.5 M HCl. All reactors were treated for 48 hours and were well mixed by magnetic stirrers at a constant speed of 350 rpm. The FNA concentration was calculated using the following formula.

$$FNA(mg\ HNO_2\text{-}N\ L^{-1}) = S_{NO2^-\_N}/(K_a \times 10^{pH}) \quad \text{Eq. 1}$$

where $S_{NO2^-\_N}$ is the dissolved NO_2^- concentration (mg N L^{-1}) and $K_a = e^{-2{,}300/(273.15+T)}$; temperature T (ºC) for this study was 25 °C (Anthonisen et al., 1976).

In each experiment, mixed liquor samples were taken before and after the pre-treatment using a syringe and immediately filtered through disposable Millipore filter units (0.22 μm pore size) for the off-line analysis.

Table 2: FNA pre-treatment and BMP tests conditions.

FNA pre-treatment	**NO_2^- concentration (mg N L^{-1})**	**FNA concentration (mg NO_2-N L^{-1})**
FNA-control	0	0
FNA0.77	100	0.77
FNA2.31	300	2.31
FNA14.61	1900	14.61
BMP tests	**Substrate source**	**Initial NO_2-N concentration (mg N L^{-1})**
BMP-control	Algae from FNA-control	0
BMP1	Algae from FNA2.31 with liquor removal	0
BMP2	Algae pre-treated liquor from FNA0.77	53
BMP3	Algae pre-treated liquor from FNA2.31	159
BMP4	Algae pre-treated liquor from FNA14.61	1006
BLK-I*	-	0
BLK-II*	-	53
BLK-III*	-	1006

* No algae was added to the BLK series.

2.3. Anaerobic batch biochemical methane potential tests

The biochemical methane potential (BMP) assay provides a measure of the anaerobic biodegradability of a given substrate, and was used to determine the methane yields and process kinetics of the algal biomass with and without FNA pre-treatment (0-14.61 mg HNO_2-N L^{-1}, for 60 h), as described in Table 2. Algal biomass was batch digested in 240 mL sealed glass serum bottles (170 mL working volume). Batches were performed at an inoculum to substrate ratio of 1.5 (VS basis), where each BMP test contained 90 mL algal liquor and 80 mL inoculum. The digested sludge, used as inoculum, was degassed and kept under anaerobic conditions at 38±1 °C for 6 days prior to commencing the experiments. The anaerobic conditions were established by flushing the headspace of each serum bottle with high purity nitrogen immediately followed by sealing with butyl rubber stopper secured by an aluminium crimp cap. The batches were incubated at 38±1 °C and agitated every 2-3 days. As shown in Table 1, the substrate of BMP-control was the algal liquor of the FNA-control trial; the substrate of BMP 1 was the algal pellet after FNA2.31 pre-treatment with the liquid removal by centrifuge; the substrate of BMP 2-4 was the pre-treated algae from FNA0.77, FNA2.31, and FNA14.61, respectively. Three sets of blanks (BLK-I, -II and -III) were also set up. BLK-I contained inoculum and Milli-Q water without algae. BLK-II and -III were identical to BLK-I except with the addition of nitrite stock solution, which resulted in an initial nitrite level of around 53 and 1006 mg N L^{-1} in BLK-II and -III, respectively. This was to evaluate the effect of nitrite on the performance of the inoculum. All tests were carried out in triplicates. The BMP tests lasted for 110 days, when biogas production dropped to insignificant levels. The biogas (CH_4, CO_2, H_2) production was monitored by subtracting biogas production from corresponding blanks. The methane yields were reported both on a COD basis (g CH_4-COD per g COD added) and a VS basis (L CH_4 per kg VS added) to make it easily comparable literatures.

2.4. Biochemical methane potential tests modelling

A two-substrate model was applied in this study (Eq. 2). The key parameters associated with methane production from algae, the hydrolysis rates and the biodegradability, were modelled to evaluate and compare methane production rate and yield.

$$B(t) = B_{0,rapid}\left(1 - e^{-k_{rapid}t}\right) + B_{0,slow}\left(1 - e^{-k_{slow}t}\right) \quad \text{Eq. 2}$$

where $B_{0,rapid}$ is the biochemical potential of the rapidly biodegradable substrates (g CH_4-COD per g COD added); $B_{0,slow}$ is the biochemical potential of the slowly biodegradable substrates

(g CH_4-COD per g COD added); k_{rapid} is the hydrolysis rate of the rapidly biodegradable substrates (d^{-1}); and k_{slow} is the hydrolysis rate of the slowly biodegradable substrates (d^{-1}).

The parameters were determined by fitting the cumulative methane production data (average data from triplicate tests) from BMP tests to the kinetic model. The model was implemented in a modified version of Aquasim 2.1d (Batstone et al., 2009).

2.5. Analysis

The TS, VS, TCOD and SCOD were measured according to standard methods (American Public Health Association, 2005). TCOD and SCOD were measured using Spectroquant® photometric cell tests (114541 and 114555, Merk, Germany), a Thermoreactor TR 300 (Merck, Germany) and a UV-Visible Spectrophotometer (Varian Cary®50, Varian, Inc., Australia). NH_4^+-N, NO_3^--N, NO_2^--N, PO_4^{3-}-P were measured with a Lachat QuikChem 8000 Flow Injection Analyser (Lachat Instrument, Milwau-kee, USA). The protein concentration was measured by the bicinchoninic acid (BCA) method with Bovine serum albumin (BSA) as standard (Smith et al., 1985). The carbohydrate concentration was determined by the Anthrone method with glucose as standard (Raunkjær et al., 1994). The lipid content was measured by an InfraCal TOG/TPH Analyzer (Wilks Enterprise, Inc., USA) using S-318 as the extraction solvent.

Microscope observation and cell membrane damage assays were performed before and after pre-treatment. Untreated algae and algae from FNA2.31 pre-treatment was diluted 10 times before staining. The SYTOX Green fluorescent probe (Invitrogen, Ltd., UK) was supplied as a 5 mM stock solution in DMSO. 0.5 µL of this stock solution was added to 0.5 mL cell suspension giving a final dye concentration of 5 µM, and the mixture was incubated for 5 minutes at room temperature in the dark. Algal cells in 24 random fields were counted under a fluorescence microscope (BX61, Olympus, Tokyo, Japan) equipped with a double band pass filter set at 473-498 and 548-573 nm for excitation, and at 515-535 and 590-620 nm for emission. Images were obtained and post-adjusted with DPC controller 1.2.1 (Olympus optical co. LTD.) and iTEM 5.0 (Olympus Soft Imagining Solutions, Olympus optical co. LTD.) respectively.

The composition of biogas produced during digestion was measured with a Perkin-Elmer loop injection GC. The Perkin-Elmer GC-TCD (AutoSystem GC, Perkin-Elmer, Waltham, MA, USA) is fitted with a 2.44 m stainless steel column (Haysep at 80/100 mesh) and a GC Plus Data Station (model 1022, Perkin-Elmer, Waltham, MA, USA). High purity nitrogen was used as carrier gas at a

flow pressure of 55 kPa. The injection port temperature was 75 °C, the oven temperature at 40 °C and the detector temperature at 100 °C (American Public Health Association, 2005). The GC was calibrated using external standard gases from British Oxygen Company (Sydney, NSW, Australia).

3. Results and Discussion

The objectives of the work are to i) evaluate the effect of FNA pre-treatment on methane yield and kinetics of digestion and ii) understand the mechanism of FNA pre-treatment, and accordingly understand the performance improvements by FNA pre-treatment.

3.1. Methane yield and kinetics of anaerobic digestion

Biochemical methane potential (BMP) tests are used to evaluate anaerobic biodegradability, which is reported as the ultimate methane potential per mass of substrate ($B_{0,\,total}$). BMP tests are also used to investigate process kinetics.

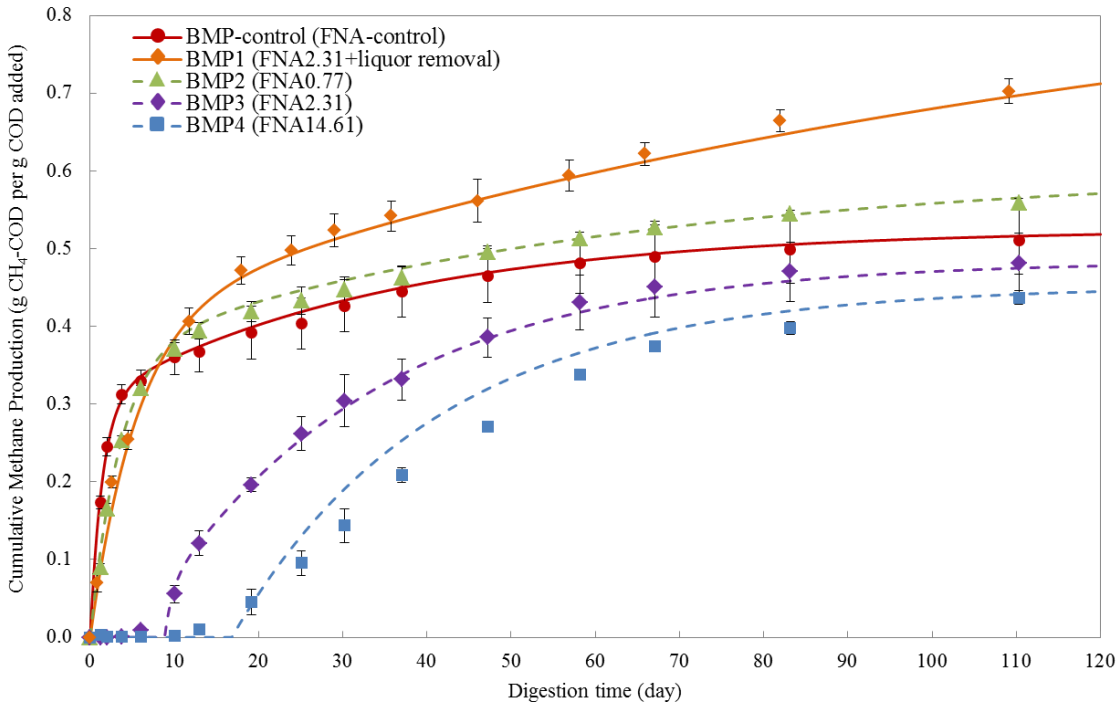


Figure 1: Cumulative methane production (g CH_4-COD per g COD added) from algae. Error bars show standard error in triplicate tests and lines show model predicted trends using two-substrate model.

Kinetic analysis was applied to help understand the effect of FNA pre-treatment on the algae digestion. Algae apparently consists of rapidly and slowly degradable components, with slowly degradable components including polymers and structurally complex parts of the cells (Bohutskyi et al., 2014). The hydrolysis of these slowly degradable components is the rate-limiting step of anaerobic digestion (Eastman & Ferguson, 1981). Therefore, a two-substrate model was applied in this study. The simulated methane production curves using the two-substrate model are shown in Figure 1. The figure shows methane production was well predicted by the two-substrate model.

The biochemical methane potentials ($B_{0,rapid}$ and $B_{0,slow}$) and reaction rates (k_{rapid} and k_{slow}) are shown in Table 3. Comparing BMP1 to BMP-control shows that FNA2.31 pre-treatment dramatically enhanced the methane production yields with an increase in both in $B_{0,rapid}$ and $B_{0,slow}$ observed. With 2.31FNA pre-treatment, $B_{0,rapid}$ and $B_{0,slow}$ increased to 0.40 and 0.43 g CH_4-COD per g COD added compared to 0.30 and 0.22 g CH_4-COD per g COD added in the case of untreated algae (BMP-control).

Comparing BMP2-4 to BMP-control shows that higher FNA concentrations were counterproductive, with methane potentials ($B_{0,total}$) for the high FNA pre-treatments being less than for the BMP control.

In contrast to ultimate biodegradability, the reaction rates k_{rapid} and k_{slow} both decreased after FNA pre-treatment, likely due to multiple factors, including inhibition. The removal of FNA pre-treatment liquor implies a drop in $B_{0,rapid}$. But $B_{0,rapid}$ actually increased after 2.31FNA pre-treatment (Table 3), which was probably because these components are present in the algal cells. Since the accessibility of AD microorganisms to $B_{0,rapid}$ played a decisive role in the process rate, it is likely that the accessibility to $B_{0,rapid}$ resulted in a decrease in $k_{0,rapid}$. The reasons for the reduction in rates are discussed in the following section in details.

Table 3: Estimated k_{rapid}, $B_{0,rapid}$, and k_{slow}, $B_{0,slow}$, t_d using two-substrate model (with 95 % confidence intervals). Calculated $B_{0,total}$ and yields of BMP tests with and without FNA pre-treatment (mean ± SE). SE shows standard errors of triplicate tests.

Sample	k_{rapid} (d^{-1})	$B_{0,rapid}$ (g CH_4-COD per g COD added)	k_{slow} (d^{-1})	$B_{0,slow}$ (g CH_4-COD per g COD added)	t_d	$B_{0,total}$ (g CH_4-COD per g COD added)	Overall yield (Y_O)			Anaerobic digestion yield (Y_{AD})		
							COD basis (g CH_4-COD per g TCOD added)	VS basis (L CH_4 per kg total VS added)	Increase /Decrease [a] (%)	COD basis (g CH_4-COD per g COD added to AD)	VS basis (L CH_4 per kg VS added to AD)	Increase /Decrease [a] (%)
BMP-control	0.70±0.08	0.30±0.01	0.026±0.01	0.22±0.01	-	0.53±0.01	0.53±0.01	161±7	-	0.53±0.01	161±7	-
BMP1	0.22±0.03	0.40±0.02	0.012±0.002	0.43±0.02	-	0.82±0.02	0.80±0.02	250±2	53	0.82±0.02	254±1	55
BMP2	0.29±0.02	0.25±0.03	0.019±0.04	0.25±0.01	-	0.50±0.03				0.50±0.03		
BMP3	0.14±0.02	0.086±0.03	0.034±0.002	0.42±0.03	8.9±2	0.51±0.03				0.51±0.03		
BMP4	0.10±0.02	0.023±0.01	0.039±0.006	0.44±0.01	17±1	0.46±0.01				0.46±0.01		

[a] compared to the BMP-control, on COD basis.

Boost in methane yield by FNA pre-treatment

The effect of FNA pre-treatment on algae methane yields was evaluated by comparing methane production from BMP1 and BMP-control. The substrate of BMP1 was the algal biomass collected after 2.31 mg NO_2-N L^{-1} FNA pre-treatment. This FNA concentration was chosen based on a previous study where it was demonstrated that after 2.31 mg NO_2-N L^{-1} FNA pre-treatment, algal cell disruption was severe (Bai et al., 2014). Since nitrite has been shown to be toxic to methanogenesis during anaerobic digestion (Banihani et al., 2009), the pre-treatment liquor was removed to eliminate the influence of nitrite in the liquor on anaerobic digestion.

In order to evaluate the effect of FNA pre-treatment on the methane yield from anaerobic digestion, two yields were determined from the experiments, namely the anaerobic digestion yield (Y_{AD}) and the overall yield (Y_O), as shown in Figure 2. Y_{AD} is methane produced per mass of substrate introduced into the digester. It represents the yield of the anaerobic digestion process. Y_O is the methane produced per mass of substrate introduced into the system (before FNA pre-treatment), and so represents the yield of the overall process.

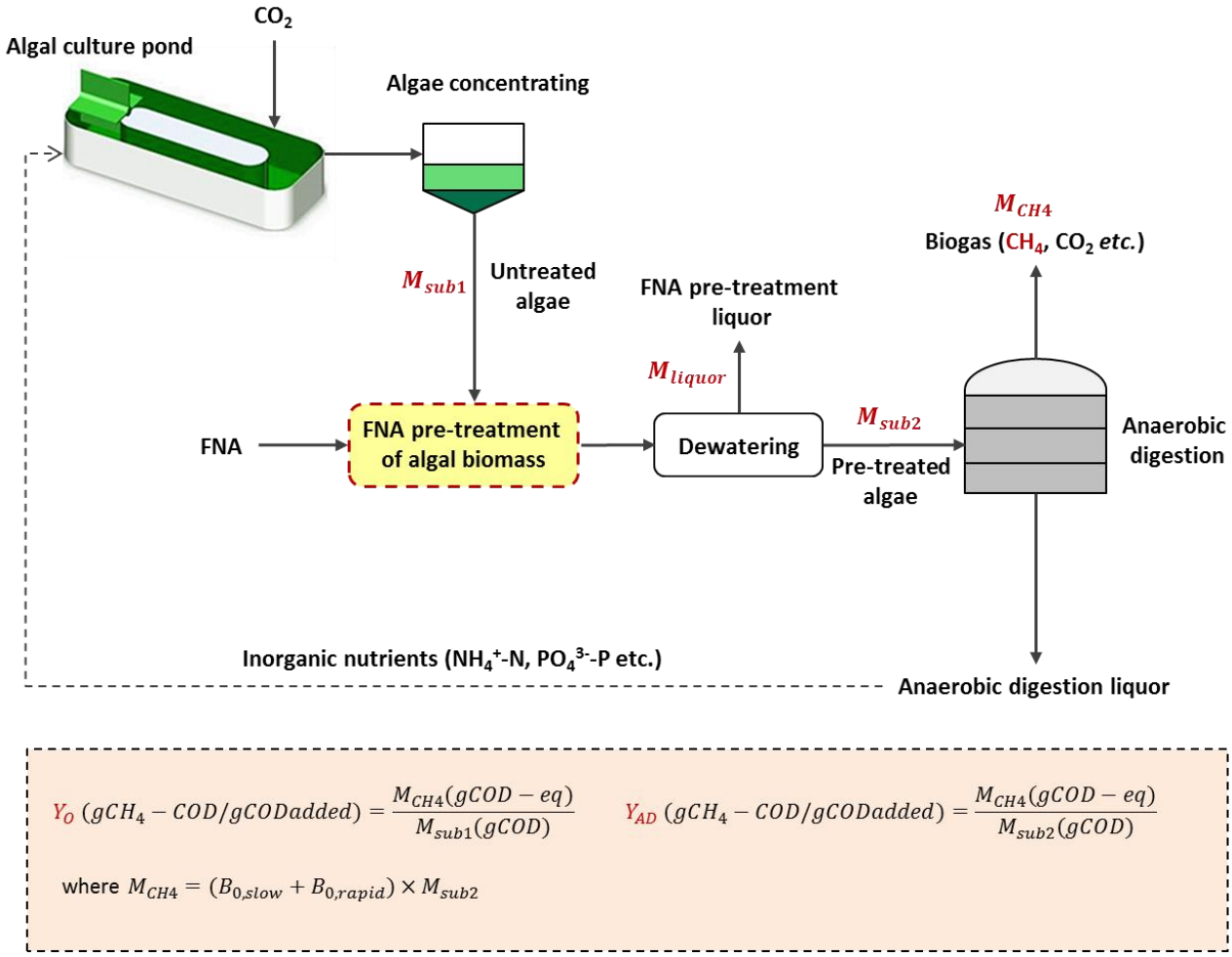


Figure 2: Proposed FNA pre-treatment technology for enhancing methane production from algae.

As shown in Table 3, the overall methane yields from untreated algae (BMP-control) and FNA pre-treated algae (BMP1) observed in this study are relatively low when compared to those reported on *Tetraselmis* sp. in other studies, ranging between 100 and 400 L CH_4 per kg VS added (Bohutskyi et al., 2014; Marzano et al., 1982). This was expected as the algal biomass was not enriched for lipids (which resulted in low C/N ratio in algal biomass) and the algae had a high culture age (retention time of 15 days). Both these factors have been known to decrease the methane yields (Uggetti et al., 2014; Venkata Mohan et al., 2008).

Comparing BMP1 to BMP-control, the FNA2.31 pre-treatment increased the methane yield dramatically, with a 55 % increase in Y_{AD} and a 53 % increase in Y_O. This could be mainly due to the

cell disruption by FNA pre-treatment. This hypothesis is in line with the conclusion of the study by Bai et al. (2014) that the cell disruption effect generated by FNA pre-treatment enhanced the total lipid extraction yield from algae.

Contribution of FNA pre-treatment liquor

Including the FNA pre-treatment liquor with the substrate needs careful consideration because the liquor has both positive and negative effects on the methane production. After FNA pre-treatment, the composition of the solubilised organic matter in the pre-treatment liquor was mainly determined as soluble protein and polysaccharides (see Section 3.2.). These compounds are beneficial to methane production in anaerobic digestion since they are rapidly biodegradable components which can improve the methane production (Wang et al., 2013).

The pre-treatment liquor into the digestion did not enhance the methane yield. Rather, the overall methane yields decreased 4-13 % for BMP2-4 compared to the BMP-control. Two of these negative effects are: (i) denitrification of NO_2^- and (ii) toxicity of FNA to methanogens.

It is known that NO_2^- is a preferred electron acceptor (Banihani et al., 2009). Table 4 shows the COD requirements for nitrite reduction and growth of denitrifiers. These COD requirements are one of the causes for the obvious delay of methane production in BMP3 and BMP4.

Table 4: COD requirements for nitrite reduction (through denitrification) and growth of denitrifiers for nitrite-amended culture used in this study.

BMP	Nitrite concentration (mg N L^{-1})	COD required (mg COD L^{-1})		
		Denitrification (mg COD L^{-1} [a])	Growth of denitrifiers (mg COD L^{-1} [b])	Total (mg COD L^{-1})
BMP2	53	91	176	267
BMP3	159	273	529	802
BMP4	1006	1724	3350	5074

[a] Calculated based on 1.714 mg COD mg NO_2-N^{-1};

[b] Calculated based on theoretical f_e and f_s values, which were 0.34 and 0.66, respectively, neglecting microbial decay. f_e and f_s are the fractions of the electron donor (electron equivalent basis) used for energy generation (i.e., nitrite reduction) and microbial growth, respectively (Tugtas & Pavlostathis, 2007).

The other drawback of carrying pre-treatment liquor into the digester is that nitrite present in the pre-treatment liquor has potential toxicity to methanogenesis (Banihani et al., 2009; Chen et al., 2008). Methane production from BLK-II (lower nitrite concentration) was slightly delayed compared to BLK-I (no nitrite); methane production from BLK-III (upper nitrite concentration) was completely inhibited through the entire BMP tests. Banihani *et al.* (2009) revealed that 95 % or greater inhibition of methanogenesis was evident at the lowest concentrations of added NO_2^- tested (8 mg N L^{-1}); and the recovery was only partial at high NO_2^- concentrations (62-153 mg N L^{-1}). The inhibition/toxic effect of NO_2^- was severe in our study which is in line with other reported works (Balderston & Payne, 1976; Tugtas & Pavlostathis, 2007). Based on the study of Banihani et al. (2009), methanogenesis was inhibited completely in the present of NO_2^- (8-153 mg N L^{-1}) ; and it started once the NO_2^- was consumed.

The negative effects of nitrite need to be taken into account when considering the kinetic data. The k_{slow} and k_{rapid} in the two-substrate model are lumped parameters, affected by substrate availability, inhibition, physical access to substrate, and even cell counts. The factors contributed to reduced k_{slow} and k_{rapid} for the FNA systems in most cases, particularly for k_{rapid}, which reflects the kinetics through the early stages of digestion, when the presence of nitrite would be most significant.

3.2. Mechanism of FNA pre-treatment

The BMP tests showed that FNA pre-treatment can result in a boost in both $B_{0,rapid}$ and $B_{0,slow}$, and consequently a boost in Y_{AD}. It is suspected that this is due to the pre-treatment disrupting cell walls and even intracellular membranes. Both can act as barriers to methanogenic activity on encapsulated organics. Disruption of the algal cells was visualized under the fluorescent microscope after SYTOX Green staining (Figure 4). SYTOX Green has a high affinity to nucleic acid in the cells and can emit bright green fluorescence light when excited with a 450-490 nm source (Sato et al., 2004). Since it is a large molecule, SYTOX Green could only penetrate into membrane damaged cells. Autofluorescence of chlorophyll was observed simultaneously, which allowed the discrimination of membrane damaged cells (green colour) and intact cells (red colour). BMP-control algae exhibited undisrupted cells with only a few percentage of disrupted ones; nevertheless, after FNA2.31 pre-treatment, almost all cells were stained with green colour clearly indicating cell membrane disruption.

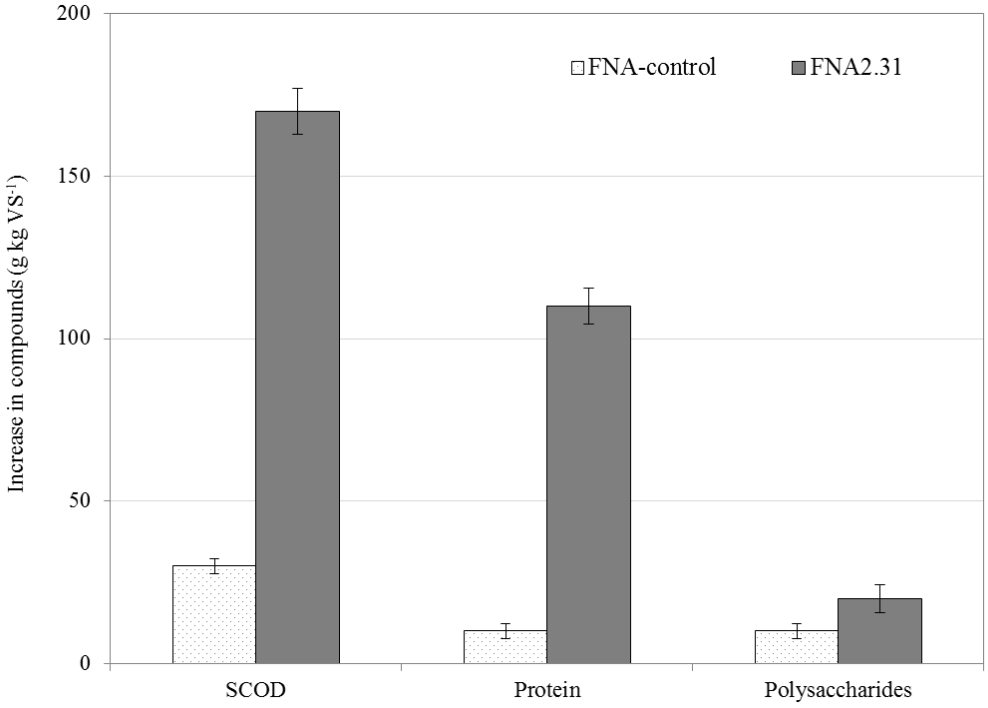


Figure 3: FNA pre-treatment on algal biomass solubilisation in terms of SCOD, protein and polysaccharides concentration.

Disrupting cells led to a boost in soluble substrates, likely due to release of organics. Figure 3 shows the increase in SCOD, soluble protein and polysaccharides concentration after the FNA2.31 pre-treatment, compared to the changes from BMP-control. As shown in Figure 3, solubilisation of organic matter (SCOD) increased 5.7 times more after FNA2.31 pre-treatment compared to the control. The characterisation of the soluble organic matter shows that the SCOD mainly consisted of protein and polysaccharides. After algae was treated at an FNA level of 2.31 mg HNO_2-N L^{-1}, soluble protein and polysaccharides concentrations in the pre-treatment liquor increased from 10.2 to 110 g kg VS^{-1} and 10.1 to 20.5 g kg VS^{-1}, respectively.

But the boost in soluble substrates is far outweighed by the increase in $B_{0,slow}$. The increase in $B_{0,slow}$ is a unique and important result. Considering FNA pre-treatment of waste activated sludge (WAS), availability of slowly biodegradable substrates seemed to be unaffected by FNA pre-treatment (Wang et al., 2013). But this work shows that for algae, $B_{0,slow}$ can be doubled with FNA pre-treatment (BMP1 compared to BMP-control). For algal cells, the rigid cell envelope and even intracellular structures are major barriers to effective digestion (Bohutskyi et al., 2014). Disrupting the cells and intracellular compartment enables access to internal organics. This supports our previous work on extracted lipids from algae which found that solvent penetration in FNA treated algae is far more effective than in untreated algae (Bai et al., 2014).

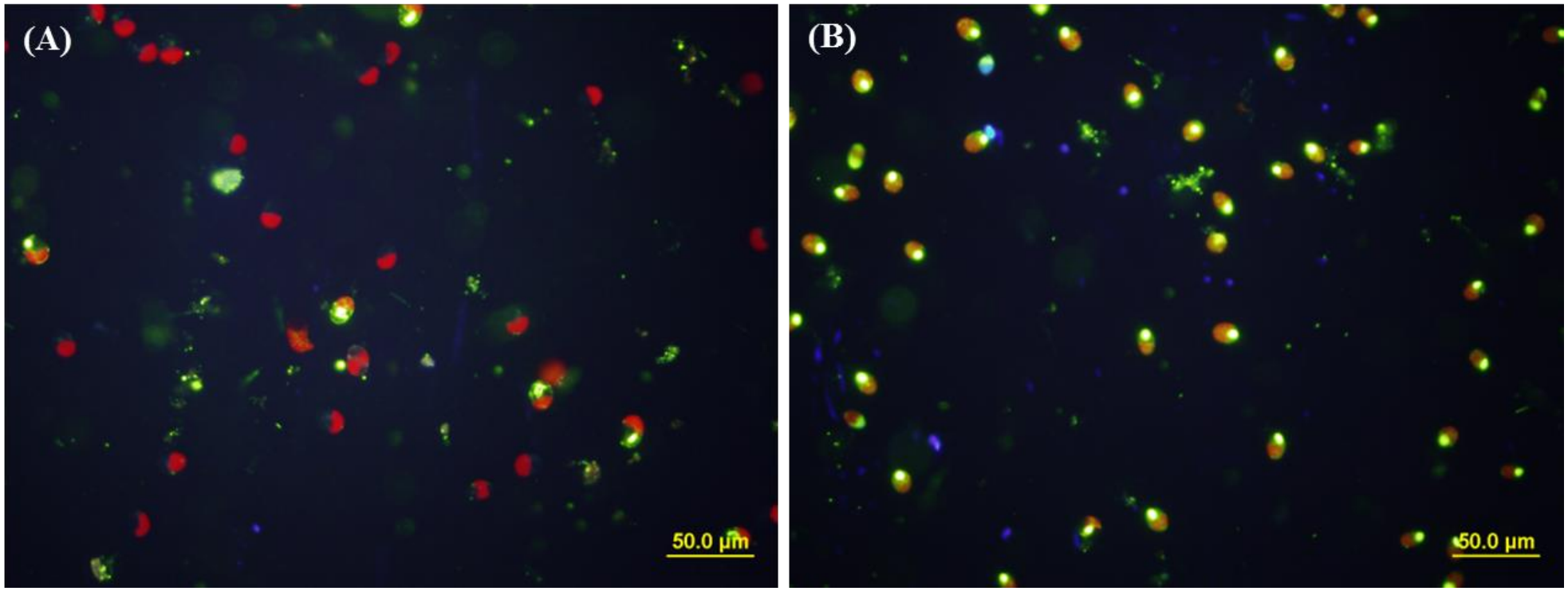


Figure 4: Sytox Green staining fluorescent microscope images of (A) untreated algae and (B) FNA-2.31 pre-treated algae.

4. Conclusions

The methane production yield from algae through anaerobic digestion was found to be dramatically enhanced by FNA pre-treatment (2.31 mg HNO_2-N L^{-1}), with a 55 % increase in Y_{AD} and a 53 % increase in Y_O. Plus, detailed kinetic analysis of methane production from BMP test based on model simulation indicates that with FNA2.31 pre-treatment, $B_{0,rapid}$ and $B_{0,slow}$ increased to 0.40 and 0.43 g CH_4-COD per g COD added comparing to 0.30 and 0.22 g CH_4-COD per g COD added in the case of untreated algae (BMP-control). Cell disruption effect of FNA pre-treatment contributes to the improved performance of anaerobic digestion process. It was concluded that FNA pre-treatment technique would be an efficient method for enhancing methane production from algal biomass through anaerobic digestion.

Acknowledgements

The authors wish to thank Prof. Peer Schenk and Mr. Rakesh Narala of the Algae Biotechnology Group (The University of Queensland, Australia), for providing the algal inoculum and culturing protocols.

Reference

American Public Health Association. 2005. Standard methods for the examination of water and wastewater. APHA-AWWA-WEF, Washington, DC.

Anthonisen, A.C., Loehr, R.C., Prakasam, T.B.S., Srinath, E.G. 1976. Inhibition of Nitrification by Ammonia and Nitrous Acid. Journal (Water Pollution Control Federation), 48(5), 835-852.

Bai, X., Ghasemi Naghdi, F., Ye, L., Lant, P., Pratt, S. 2014. Enhanced lipid extraction from algae using free nitrous acid pretreatment. Bioresource Technology(0).

Balderston, W.L., Payne, W.J. 1976. Inhibition of methanogenesis in salt marsh sediments and whole-cell suspensions of methanogenic bacteria by nitrogen oxides. Applied and Environmental Microbiology, 32(2), 264-269.

Banihani, Q., Sierra-Alvarez, R., Field, J. 2009. Nitrate and nitrite inhibition of methanogenesis during denitrification in granular biofilms and digested domestic sludges. Biodegradation, 20(6), 801-812.

Batstone, D.J., Tait, S., Starrenburg, D. 2009. Estimation of hydrolysis parameters in full-scale anerobic digesters. Biotechnology and Bioengineering, 102(5), 1513-1520.

Bohutskyi, P., Betenbaugh, M.J., Bouwer, E.J. 2014. The effects of alternative pretreatment strategies on anaerobic digestion and methane production from different algal strains. Bioresource Technology, 155(0), 366-372.

Chen, Y., Cheng, J.J., Creamer, K.S. 2008. Inhibition of anaerobic digestion process: A review. Bioresource Technology, 99(10), 4044-4064.

Cho, S., Park, S., Seon, J., Yu, J., Lee, T. 2013. Evaluation of thermal, ultrasonic and alkali pretreatments on mixed-microalgal biomass to enhance anaerobic methane production. Bioresource Technology, 143(0), 330-336.

Eastman, J.A., Ferguson, J.F. 1981. Solubilization of particulate organic carbon during the acid phase of anaerobic digestion. Journal (Water Pollution Control Federation), 352-366.

Keymer, P., Ruffell, I., Pratt, S., Lant, P. 2013. High pressure thermal hydrolysis as pre-treatment to increase the methane yield during anaerobic digestion of microalgae. Bioresource Technology, 131(0), 128-133.

Mahdy, A., Mendez, L., Ballesteros, M., González-Fernández, C. Enhanced methane production of Chlorella vulgaris and Chlamydomonas reinhardtii by hydrolytic enzymes addition. Energy Conversion and Management(0).

Marzano, C.M.A.D.S., Legros, A., Naveau, H.P., Nyns, E.J. 1982. Biomethanation of the Marine Algae Tetraselmis. International Journal of Solar Energy, 1(4), 263-272.

Passos, F., Hernández-Mariné, M., García, J., Ferrer, I. 2014. Long-term anaerobic digestion of microalgae grown in HRAP for wastewater treatment. Effect of microwave pretreatment. Water Research, 49(0), 351-359.

Ras, M., Lardon, L., Bruno, S., Bernet, N., Steyer, J.-P. 2011. Experimental study on a coupled process of production and anaerobic digestion of Chlorella vulgaris. Bioresource Technology, 102(1), 200-206.

Raunkjær, K., Hvitved-Jacobsen, T., Nielsen, P.H. 1994. Measurement of pools of protein, carbohydrate and lipid in domestic wastewater. Water Research, 28(2), 251-262.

Richmond, A. 2004. Handbook of Microalgal Culture: Biotechnology and Applied Phycology. Wiley-Blackwell.

Sato, M., Murata, Y., Mizusawa, M., Iwahashi, H., Oka, S.-i. 2004. A simple and rapid dual-fluorescence viability assay for microalgae. Microbiol Cult Coll, 20(2), 53-59.

Sialve, B., Bernet, N., Bernard, O. 2009. Anaerobic digestion of microalgae as a necessary step to make microalgal biodiesel sustainable. Biotechnology Advances, 27(4), 409-416.

Singh, A., Olsen, S.I. 2011. A critical review of biochemical conversion, sustainability and life cycle assessment of algal biofuels. Applied Energy, 88(10), 3548-3555.

Smith, P.K., Krohn, R.I., Hermanson, G.T., Mallia, A.K., Gartner, F.H., Provenzano, M.D., Fujimoto, E.K., Goeke, N.M., Olson, B.J., Klenk, D.C. 1985. Measurement of protein using bicinchoninic acid. Analytical Biochemistry, 150(1), 76-85.

Tugtas, A.E., Pavlostathis, S.G. 2007. Inhibitory effects of nitrogen oxides on a mixed methanogenic culture. Biotechnology and Bioengineering, 96(3), 444-455.

Uggetti, E., Sialve, B., Trably, E., Steyer, J.-P. 2014. Integrating microalgae production with anaerobic digestion: a biorefinery approach. Biofuels, Bioproducts and Biorefining, n/a-n/a.

Venkata Mohan, S., Lalit Babu, V., Sarma, P.N. 2008. Effect of various pretreatment methods on anaerobic mixed microflora to enhance biohydrogen production utilizing dairy wastewater as substrate. Bioresource Technology, 99(1), 59-67.

Wang, Q., Ye, L., Jiang, G., Jensen, P.D., Batstone, D.J., Yuan, Z. 2013. Free Nitrous Acid (FNA)-Based Pretreatment Enhances Methane Production from Waste Activated Sludge. Environmental Science & Technology, 47(20), 11897-11904.